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INITIATION OF MATURATION IN THE FROG EGG

(One plate)

L. V. HEILBRUNN, KATHRYN DAUGHERTY, AND KARL M. WILBUR
University of Pennsylvania

WHEN an immature egg is induced to begin maturation, it does so as the result of a stimulus. The stimulus to maturation has often been studied in the case of invertebrate eggs; and it is well known that various types of physical and chemical agents can cause the initiation of maturation in the eggs of annelids, mollusks, etc. Recently the initiation of maturation in the egg of the annelid *Nereis* has been studied from the standpoint of the calcium-release theory of stimulation, and evidence has been presented which indicates that the breakdown of the nuclear membrane in this egg is conditioned by calcium release from the egg cortex (Heilbrunn and Wilbur, 1937).

As yet, the initiation of maturation in the frog egg is not well understood; and, as a matter of fact, the problem has scarcely been attacked on an experimental basis. It was thought that frog eggs might be studied in somewhat the same manner that invertebrate eggs have been studied. Eventually perhaps, information might be obtained which would link up the phenomena in frog and other vertebrate eggs with the facts observed and the theories proposed for invertebrate eggs. The results might also have some significance for the general protoplasmic theory of stimulation.

Immature frog eggs are easy to obtain. At most seasons of the year frog ovaries contain many large eggs, and many of these are ready to mature and to be released from the ovary. Indeed, in the case of *Rana pipiens*, Wolf (1929) and Rugh (1934) showed that implantation or injection of pituitary would cause these ovarian eggs to break through the ovarian wall and to pass into and through the oviduct. They lodge in the uterus, and they may then be squeezed out of the frog and fertilized. Presumably, therefore, the eggs which have been caused to ovulate and to pass through the oviduct as a result of pituitary treatment have begun their maturation, for normally in the frog, fertilization occurs only after maturation has begun.

In our experiments we attempted to discover the nature of the stimulus which causes the immature frog egg to begin its maturation. Our results also throw some light on the factors concerned in the release of the egg from the ovary. Rugh (1935) found that, when an excised frog ovary is immersed in Ringer's fluid, or in Ringer's fluid to which anterior

pituitary hormone has been added, the eggs remain in the ovary and do not escape. However, when he added pepsin and dilute hydrochloric acid to Ringer's fluid, such a digestive solution caused the eggs to escape in 5 minutes. Rugh therefore suggested the possibility that the anterior pituitary hormone might supply "an activator for a digestive ferment, originating in the egg and acting on the follicle wall." Since Rugh's experiments Zwarenstein (1937) has stated that addition of the mammalian hormone progesterone causes extrusion of ova from the excised ovaries of the African clawed toad, *Xenopus laevis*. The progesterone was added to Ringer's fluid in which the ovaries were bathed, and the ova were extruded in 8–10 hours.

Our results do not confirm Rugh's statement that pituitary hormone is incapable of causing the eggs to be extruded through the walls of the ovary. We prepared an extract by mashing a pituitary gland in 1 cc. of Ringer's fluid. To this was then added 29 cc. of Ringer's fluid. When ovaries were immersed in such a suspension of pituitary gland, it was noted that, after the lapse of several hours, the eggs could be observed in large numbers escaping through the wall of the ovary. For example, in experiments performed on the excised ovaries of two females (*R. pipiens*) the number of eggs extruded during 24 hours' immersion was found to be 203 and 280, respectively. Similar treatment of the excised ovaries of another female (*Rana clamitans*) resulted in the release of 101 eggs during the first 24 hours and 193 eggs during the following 24-hour period. On the other hand, when the ovaries of either species of frog were immersed in Ringer's fluid without any admixture of pituitary, no extrusion of eggs occurred. These experiments were performed in the spring of the year.

The ovarian frog egg, like immature eggs generally, possesses a large nucleus, or germinal vesicle. Our experiments are primarily concerned with the attempt to determine the stimulus to breakdown of this vesicle. Unfortunately, the eggs, as they reach their definitive size, become thoroughly opaque, so that it is not a simple matter to determine whether or not the germinal vesicle is broken down; nor can one easily follow the course of the maturation divisions. It is true that with a little practice one can dissect out the germinal vesicle from frog eggs, and in this way one might be able to decide whether the vesicle is intact. Such a method is rather uncertain, for every now and again the vesicle is injured in the dissection process, and the injured vesicles are apt to become unrecognizable. The only sure way to discover what has happened to the germinal vesicle in a given experiment is to section the eggs with ordinary histological techniques. In such sectioned eggs it is possible, without much difficulty, to make counts of intact and broken-down germinal vesicles; and one can thus determine accurately the percentage of eggs which has responded to treatment in any given experiment. The frog egg is not easy to section; and, indeed, it is well known to be unusually refractory for ordinary sectioning methods. In our studies fixation was carried out in Zenker's or Smith's fluid (see Smith, 1915). Fixation in Smith's fluid was found to be improved by reducing the acetic acid to half the amount prescribed. The sections were stained in borax carmine.

Eggs of *R. pipiens* were dissected from the ovaries in Ringer's solution. In our first experiments we tried the effect of ultra-violet radiation from a mercury arc lamp. Such radiation is very effective in inducing maturation in the egg of *Nereis*. However, no clear-cut results were obtained with the frog egg.

Obviously, therefore, the egg of the frog does not behave in the same manner as do invertebrate eggs. We were led to try the effect of pituitary extract in order to determine

whether or not this might have an effect in inducing maturation. The eggs were exposed to pituitary extracts, prepared as noted above. They were left in contact with the extracts for periods of either 24 or 48 hours. Control eggs were left in Ringer's fluid for similar periods of time. At the expiration of the experiment the eggs were sectioned, and counts were made of the percentages of intact and broken-down germinal vesicles. These counts are shown in Table 1. It is clear that no germinal-vesicle breakdown occurs in the control eggs in Ringer's fluid. On the other hand, in the eggs treated with pituitary

TABLE 1

No. of Frog	Ringer's Solution		Ringer's Solution — Pituitary Extract			
	No. of Eggs with Intact Germinal Vesicles	No. of Eggs Showing Germinal-Vesicle Breakdown	No. of Eggs with Intact Germinal Vesicles after 24 Hours' Treatment	No. of Eggs Showing Germinal Vesicle Breakdown after 24 Hours' Treatment	No. of Eggs with Intact Germinal Vesicles after 48 Hours' Treatment	No. of Eggs Showing Vesicle Breakdown after 48 Hours' Treatment
1	20	0	.....	.....	1	17
2	.....	.....	6	22	.....	.....
3	18	0	0	39	.....	.....
4	27	0	25	0	.....	.....
	22	.....	.....	.....	21	5
5	27	0	.....	.....	28	0
6	25	1*	.....	.....	10	16
7	26	0	.....	.....	3	21
8	25	0	.....	.....	16	9
9	25	0	.....	.....	16	11
10	23	0	.....	.....	17	9
11†	28	0	10	11	.....	.....
	31	.....	.....	.....	16	17
12†	28	0	25	0	.....	.....
	24	.....	.....	.....	23	2
Total	349	1*	66	72	151	107
Percentage	100	.....	47.8	52.2	58.6	41.4

* Egg abnormal in appearance.

† Holtfreter's solution was used in place of Ringer's solution.

there is a rather high percentage of breakdown. This percentage would have been higher if it were not for the fact that certain frogs contain eggs which seem to be particularly refractory to treatment. Thus, in the case of frog No. 5, not a single one of the eggs showed germinal-vesicle breakdown. The broken-down germinal vesicles often showed a characteristic appearance in the sectioned material. This is illustrated in Plate I, Figure 1. As another control, eggs from Frog No. 7 were immersed in a suspension of macerated gastrocnemius muscle and Ringer's solution. Of 32 eggs examined, all showed intact germinal vesicles after 48 hours' immersion in this suspension.

It might be thought that the breakdown of the germinal vesicle induced by pituitary extract is a pathological process which bears no relation to normal maturation. This is certainly not true, for some of the eggs treated with pituitary extract undergo maturation

divisions and show polar bodies. This is illustrated in Plate I, Figures 2 and 3, which show a polar body and two maturation spindles.

In conclusion, it may be stated with certainty that pituitary extracts are capable of initiating the maturation process in the frog egg. These extracts produce the same sort of effect that irradiation and various chemical stimulants produce on the egg of *Nereis*. Although the immature frog egg is not affected by the same stimulating agents as those which induce maturation in invertebrate eggs, it is a matter of interest that the one factor we have found to be effective for frog eggs may also be regarded as a rather general stimulating agent. At any rate, the anterior pituitary hormone causes a contraction of the smooth muscle of vertebrates. It is clear, therefore, that the initiation of maturation in the frog egg can be the result of a stimulus which can affect other types of irritable systems. As to whether in the intact frog maturation is induced as a result of the presence of pituitary extract in the blood, we offer no opinion. It is quite possible that another factor or factors may be involved.[1]

SUMMARY

Extracts of the pituitary gland of the frog cause the initiation of maturation in the frog egg. These extracts also induce the release of eggs from the ovary.

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[1] We are grateful to Dr. C. L. Parmenter for instruction in the frog-pituitary technique and for advice and help in the interpretation of the sectioned material.

PLATE I

1

2

3

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PLATE I

FIG. 1.—Section of an egg treated with pituitary extracts showing the broken-down germinal vesicle. ×60.

FIG. 2.—Section of an egg treated with pituitary extracts showing the polar body and a second maturation-division spindle (*s*). ×870.

FIG. 3.—Section of an egg treated with pituitary extracts showing the spindle. ×500.

These eggs were fixed with Zenker's fixative and stained with borax carmine.

THE RELATION OF THE MAGNESIUM ION TO ULTRA-VIOLET STIMULATION IN THE NEREIS EGG[1]

(Two figures)

KARL M. WILBUR
Zoölogical Laboratory, University of Pennsylvania

THE *Nereis* egg, after being shed into sea water, rests inert with its germinal vesicle intact until it is fertilized, after which the germinal vesicle breaks down. This breakdown of the germinal vesicle can be produced artificially by treating the egg with a variety of stimulating agents, such as ultra-violet light (Just, 1933) and isotonic solutions of NaCl and KCl. Accordingly, the *Nereis* egg provides very favorable material for the study of the mechanism of response, for one has a perfectly definite reaction, that of nuclear breakdown, which can be easily followed and which takes place in a relatively simple protoplasmic system. This reaction is apparently dependent upon the calcium ion, for, if the calcium is first removed from the egg cortex by treating the egg with sodium citrate, the stimulating agents which ordinarily produce germinal-vesicle breakdown are ineffective. It has been suggested that NaCl, KCl, and other stimulating agents act by releasing calcium from the cell cortex and that this is one step in a series of reactions leading to the breakdown of the germinal vesicle (Heilbrunn and Wilbur, 1937).

Now, it is an interesting fact that in sea water there are three cations—sodium, potassium, and calcium—which in one way or another favor stimulation; and yet in sea water the germinal vesicle does not break down spontaneously. Perhaps the fourth, and only other common cation, magnesium, actually inhibits spontaneous breakdown in sea water. It is, therefore, of interest to determine, on the one hand, the relation of magnesium to the other cations in the normal environment and, on the other, the relation of magnesium to nuclear breakdown when it is produced by a stimulating agent. Ultra-violet radiation was used as the stimulating agent, and as a quantitative index of response the percentage of nuclear breakdown was determined.

METHOD

The usual procedure employed was to transfer 1 drop of eggs in sea water to 20 cc. of the experimental solution. Approximately 1 cc. of this solution containing eggs was then placed in each of a series of dishes 3.3 cm. in diameter. After 15–20 minutes' immersion in the experimental solution the eggs were exposed to the unfiltered radiation of a mercury arc lamp at a distance of 23–24 cm. The lamp was a Uviarc Laboratory Outfit operating at 120–50 volts and 5.5–4.0 amperes.[2] The energy output was not of interest in this study and was not determined. An electric fan was used with the lamp

[1] This study was carried out under the direction of Dr. L. V. Heilbrunn. I gratefully acknowledge his kindness in offering suggestions and criticisms throughout the experimental work and the preparation of the manuscript. I am also indebted to Mr. F. J. Thomas for help with certain portions of the work.

[2] This is the same lamp used in earlier experiments (Heilbrunn and Wilbur, 1937), but the values given in that paper for voltage and amperage are not correct.

to prevent overheating of the eggs. Room temperature varied between 22° and 26°.7 C. On irradiation there was an increase in the temperature of the solution surrounding the eggs of approximately 3° with an exposure of 45 seconds. A 30-second exposure gave an elevation of approximately 2°.5, with less than 1°.5 for 10-second irradiation.

The percentage of nuclear breakdown was determined after a period of 1 hour, or longer, following irradiation. In order to make accurate counts the eggs were compressed beneath a cover slip. Each point on the graphs represents the average of ten determinations, and each of the ten determinations was made for a different individual. In every case at least one hundred eggs were counted. The characteristics of the lamp given above indicate that the energy output was not uniform throughout the experiments; in fact, slight variations could be observed from minute to minute. This, together with the variation of the material itself, would necessarily result in differences in the values obtained for different lots of eggs with the same time of exposure. However, the results for the several animals of each experiment were strikingly consistent in that they were always in the same direction. And there is no instance in which any one lot of eggs behaved in a different manner from those of the rest of the series.

RESULTS

Figure 1 indicates the inhibiting action of $MgCl_2$ when eggs are irradiated in a solution consisting of 1 part isotonic $MgCl_2$ to 4 parts of sea water. It will be noted that, when eggs are irradiated 10 seconds in a solution containing excess magnesium, there is not nearly so much germinal-vesicle breakdown as on irradiation in sea water. However, with a longer time of irradiation (20 and 30 seconds) the $MgCl_2$ does not inhibit the action of ultra-violet radiation. This is similar to the reaction which other systems show in the presence of anesthetics, in that a response is obtained if the stimulus is sufficiently increased.

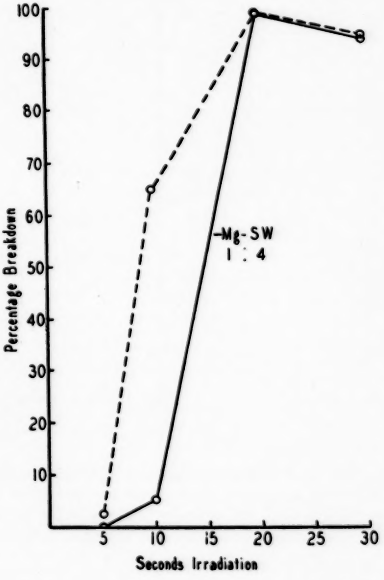

FIG. 1.—The percentage of germinal-vesicle breakdown following different periods of ultra-violet irradiation. The solid line gives results for eggs in a mixture of 1 part of isotonic $MgCl_2$ to 4 parts of sea water. The broken line gives results for eggs in sea water.

If the concentration of magnesium is further increased (1 part $MgCl_2$ to 1 part sea water), the inhibitory effect is likewise increased, and the irradiation does not overcome the effect of the $MgCl_2$ even with an exposure of 30 seconds (Fig. 2). With a still further increase in the magnesium concentration (4 parts $MgCl_2$ to 1 part sea water), there is actually a greater percentage of breakdown than with the lower concentration; and with 30 seconds' irradiation the inhibitory action is almost completely overcome.

It is surprising to find that 45 seconds' irradiation in sea water produces a lower percentage of germinal-vesicle breakdown than 30 or 15 seconds' irradiation (Fig. 2).

Cytolysis, often present with shorter exposures, is extreme with 45 seconds' irradiation. However, eggs in the $MgCl_2$–sea-water mixture do now show this decrease in the percentage of germinal-vesicle breakdown. Whether these eggs in the $MgCl_2$–sea-water mixture would likewise show a decrease in percentage of breakdown if exposed longer than 45 seconds has not been determined.

When eggs are subjected to excess magnesium, they are not injured. At any rate, on return to sea water the eggs react to ultra-violet stimulation in normal fashion. Thus, the action of $MgCl_2$ is perfectly reversible. This was shown in the following manner.

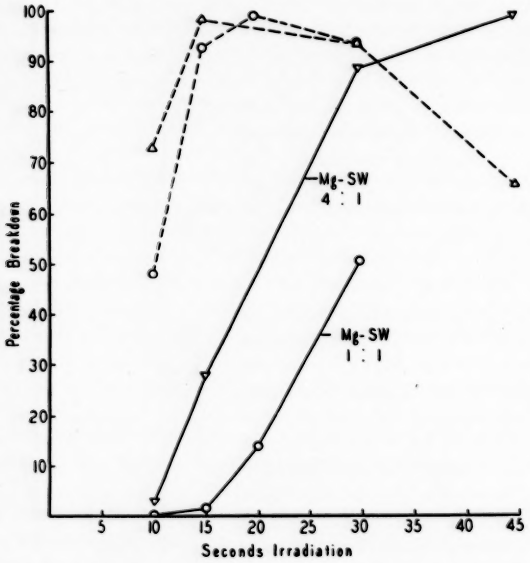

FIG. 2.—The solid line with circles indicates the percentage of germinal-vesicle breakdown following different periods of ultra-violet irradiation in a mixture of 1 part of $MgCl_2$ to 1 part of sea water. The solid line with triangles indicates results for a mixture of 4 parts of $MgCl_2$ to 1 part of sea water. The broken lines with circles and triangles indicate the corresponding sea-water controls.

Eggs were immersed in a $MgCl_2$–sea-water mixture (1 part $MgCl_2$ to 1 part sea water) for $19\frac{1}{2}$–21 minutes and then returned to sea water for a similar period. Another sample of eggs from the same female was treated only with sea water. Both samples were irradiated for 10 seconds, and the germinal-vesicle breakdown was compared. Table 1 shows that the percentage of breakdown is as great for the eggs previously treated with the $MgCl_2$–sea-water mixture as for the sea-water controls. Similar results were obtained in five experiments with a mixture consisting of 4 parts of $MgCl_2$ to 1 part of sea water. The average breakdown with 10 seconds' irradiation was 99.2 per cent for the eggs previously treated with the $MgCl_2$–sea-water mixture, as compared with an average of 97 per cent for the sea-water controls. These results eliminate the possibility that $MgCl_2$

may have inhibited the action of ultra-violet radiation by injuring the egg and thus prevented it from reacting.

The changes which take place in the egg as the result of irradiation are not instantaneous. The lifting-off of a membrane, the first obvious change following irradiation, requires a few minutes for completion. Cytolysis and other changes may occur, and after several hours some of the irradiated eggs may disintegrate.[3] Since all these

TABLE 1

REVERSIBILITY OF THE INHIBITORY EFFECT OF MAGNESIUM CHLORIDE

No. of Expt.	Percentage of Breakdown in Sea Water Following Magnesium Treatment	Percentage of Breakdown in Sea-Water Control	No. of Expt.	Percentage of Breakdown in Sea Water Following Magnesium Treatment	Percentage of Breakdown in Sea-Water Control
1	76	81	6	85	85
2	100	100	7	99	96
3	89	96	8	100	90
4	100	100	9	100	100
5	55	63	Av.	89.3	90.1

TABLE 2

INHIBITORY EFFECT OF MAGNESIUM CHLORIDE ADDED TO EGGS SUBSEQUENT TO IRRADIATION

No. of Expt.	Percentage of Breakdown Following the Addition of $MgCl_2$ to Sea Water Subsequent to Irradiation	Percentage of Breakdown in Sea-Water Control	No. of Expt.	Percentage of Breakdown Following the Addition of $MgCl_2$ to Sea Water Subsequent to Irradiation	Percentage of Breakdown in Sea-Water Control
1	0	0	6	0	0
2	0	78	7	0	17
3	0	24	8	0	38
4	0	0	9	0	36
5	0	12	10	0	35

changes require some time for completion, the possibility existed that $MgCl_2$ might inhibit germinal-vesicle breakdown if added after irradiation. To test this, eggs were irradiated for 10 seconds in sea water, and $MgCl_2$ was added 3–5 seconds later. (Four cubic centimeters of a mixture consisting of 2.5 parts of $MgCl_2$ to 1.5 parts of sea water were added to 1 cc. of sea water containing the eggs.) Table 2 shows the results. There is not a single case of breakdown in the eggs in which $MgCl_2$ was added. It is clear that the addition of $MgCl_2$ subsequent to irradiation prevents nuclear breakdown.

[3] If eggs are irradiated in sea water or a $MgCl_2$–sea-water mixture (1 part $MgCl_2$ to 1 part sea water) and counted after 1 hour and then again after several hours, the percentage of germinal-vesicle breakdown may be found to increase on standing.

In order to determine whether the customary calcium-magnesium antagonism would be exhibited by this system, eggs were irradiated for 10 seconds in a $MgCl_2$–sea-water mixture containing a quantity of $MgCl_2$ sufficient to inhibit germinal-vesicle breakdown and compared with eggs irradiated in a mixture containing the same amount of $MgCl_2$ but with $CaCl_2$ added to give the calcium-magnesium ratio found in sea water (Table 3). With the $MgCl_2$–sea-water mixture there is the usual absence of breakdown. But when $CaCl_2$ is added, the percentage of breakdown is increased, although not as much as in the sea water alone. There is thus evidence of a calcium-magnesium antagonism.

TABLE 3

THE COUNTERACTING EFFECT OF CALCIUM CHLORIDE ON THE INHIBITORY ACTION OF MAGNESIUM CHLORIDE

No. of Expt.	Percentage of Breakdown in Sea-Water–$MgCl_2$ Solution	Percentage of Breakdown in Sea-Water–$MgCl_2$–$CaCl_2$ Solution	Percentage of Breakdown in Sea Water	No. of Expt.	Percentage of Breakdown in Sea-Water–$MgCl_2$ Solution	Percentage of Breakdown in Sea-Water–$MgCl_2$–$CaCl_2$ Solution	Percentage of Breakdown in Sea Water
1.....	0	21	99	9.....	2	98	100
2.....	0	49	98	10.....	0	0	39
3.....	1	40	92	11.....	0	11	99
4.....	0	15	100	12.....	0	0	12
5.....	0	19	96	13.....	0	47	100
6.....	0	0	63	14.....	0	2	89
7.....	0	0	73	15.....	1	90	100
8.....	0	0	43				
				Av...	0.3	26.1	80.2

Since magnesium in a concentration higher than that found in sea water inhibits germinal-vesicle breakdown resulting from ultra-violet irradiation, one might logically suppose that the magnesium present in normal sea water is likewise exerting an inhibitory action. If such is the case, it should be true that eggs in sea water lacking magnesium will be more sensitive than eggs in sea water containing the normal amount of magnesium. To test this hypothesis, eggs were irradiated in artificial sea water which did not contain magnesium, and were then compared with eggs irradiated in artificial sea water containing magnesium.[4] An irradiation time of 4 seconds was employed in order that there might be no germinal-vesicle breakdown in the eggs irradiated in the artificial sea water containing magnesium. From the results shown in Table 4 it seems clear that magnesium in the concentration in which it occurs in sea water tends to inhibit the response to ultra-violet stimulation. In the absence of magnesium the sensitivity of the eggs is increased.

In four out of the ten cases a few of the control eggs in the magnesium-free sea water which had not been irradiated showed germinal-vesicle breakdown and irregular cleavage, the percentages of breakdown being 1, 4, 13, and 16. In other words, in the absence

[4] The formula given by Howard (1931) was used. In the magnesium-free sea water, NaCl was substituted in amount osmotically equivalent to the $MgSO_4$ and $MgCl_2$. A pure isotonic solution of NaCl is known to have a stimulating action on the *Nereis* egg; and it is possible that the substituted NaCl, though small in amount as compared with the amount of this salt normally present in sea water, may not be entirely without effect.

of magnesium a portion of the eggs shows a spontaneous breakdown of the germinal vesicle. In view of the low pH values for the magnesium-free and Howard sea water (5.70 and 5.84, respectively), it was thought advisable to repeat the observations in more alkaline media. Eggs were placed in magnesium-free and Howard's sea water adjusted to pH 8.0–8.2 with NaOH.[5] In each of the seven experiments performed, most of the eggs in the magnesium-free medium showed formation of two polar bodies, and some underwent irregular cleavage or lobulation (cf. Dalcq, 1924). All controls in the sea

TABLE 4

GERMINAL-VESICLE BREAKDOWN FOLLOWING 4 SECONDS' IRRADIATION

No. of Expt.	Percentage of Breakdown in Magnesium-Free Sea Water	Percentage of Breakdown in Sea Water Containing Magnesium	No. of Expt.	Percentage of Breakdown in Magnesium-Free Sea Water	Percentage of Breakdown in Sea Water Containing Magnesium
1	99	0	6	100	0
2	100	0	7	97	0
3	100	0	8	45	0
4	99	0	9	78	0
5	97	0	10	100 (count uncertain)	0

water containing magnesium had intact germinal vesicles. Similar results were obtained in two repetitions of the experiment using Wheeler's formula for sea water (1910). Such sea water contains $CaCO_3$ and showed a pH of 7.68.

DISCUSSION

The *Nereis* egg may be considered as a delicately poised system—a system which may be thrown out of its resting state by any one of a number of stimulating agents. A stimulating agent, in impinging upon the surface of the cell, initiates a series of reactions which lead to the breakdown of the germinal vesicle; and in certain cases these reactions continue on and bring about the formation of polar bodies and irregular cleavage. An explanation of the mechanism of this series of reactions in the *Nereis* egg would presumably find wide application, for at least some of these processes take place whenever germ cells maturate and cell division occurs.

This succession of changes can be inhibited or interrupted and the breakdown of the germinal vesicle thus prevented. This has been done in the present study in two ways: by the use of $MgCl_2$ and by prolonging the period of irradiation.

The *Nereis* egg is similar to other protoplasmic systems in that magnesium reversibly inhibits its response to a stimulating agent. Indeed, the egg may be said to undergo magnesium anesthesia. Heilbrunn (1937, chap. xxxvii) has suggested the following explanation for magnesium anesthesia. The primary effect of a stimulating agent is the release of calcium from a calcium-proteinate gel in the cell cortex. The free calcium then

[5] This alkalinized sea water contains no buffer, and the pH is easily changed by contact with air. Thus, samples taken from the magnesium-free and Howard sea water in which the eggs were immersed showed pH values of 6.96 and 6.84, respectively.

initiates a series of reactions (including a protoplasmic clotting), and these reactions constitute the response of the cell. With magnesium anesthesia the calcium in the cortex is replaced by magnesium, and, on stimulating, magnesium is released instead of calcium. Magnesium is much less potent than calcium in causing protoplasmic clotting; it is for this reason that the response fails to take place. That a replacement of calcium by magnesium in the cortex previous to stimulation is not necessary is shown by the result that the egg with its normal cortex can be irradiated in sea water and the response inhibited by the addition of $MgCl_2$ after irradiation has ceased. However, this does not exclude the possibility of an exchange between calcium and magnesium in the cortex following irradiation.

The importance of calcium in the process of germinal-vesicle breakdown has been suggested by several investigators (Dalcq, 1925, 1928; Hörstadius, 1923; Hobson, 1928; Pasteels, 1935); but, unfortunately, little is known of its mode of action with respect to this process. Quite possibly the inhibitory action of magnesium is due to interference with the action of calcium. Perhaps magnesium exerts an inhibitory action merely because it is less potent than calcium, as Heilbrunn suggests. If this is true, possibly a sufficient increase in the proportion of magnesium might then compensate for this difference in potency, and the response would take place. The fact that a mixture of 4 parts of $MgCl_2$ and 1 part of sea water has less effect in inhibiting germinal-vesicle breakdown than a mixture of equal parts of $MgCl_2$ and sea water would seem to lend some support to this interpretation. Pasteels (1935) has found a similar relationship for the breakdown of the germinal vesicle of the egg of *Asterias glacialis* in $MgCl_2$–sea-water mixtures. When this egg is shed into sea water, the germinal vesicle normally breaks down. However, the percentage of breakdown is inhibited progressively as the proportion of $MgCl_2$ is increased up to a certain concentration. Further increase in the proportion of $MgCl_2$ gives less inhibition, and in pure $MgCl_2$ the breakdown is as great as in sea water.

In Pasteel's work and in the present study the breakdown is partially determined by the calcium-magnesium balance. The spontaneous breakdown of the germinal vesicle observed in sea water lacking magnesium is believed to be due in part to a disturbance of this balance. We might assume that, in a magnesium-free medium, magnesium leaves the cell (cf. Ikedo, 1937) and that free calcium, in entering to establish equilibrium, increases the proportion of free calcium. The excess calcium would then initiate the process leading to germinal-vesicle breakdown; or bound calcium may be released within the cell, and this calcium might in itself be sufficient to initiate germinal-vesicle breakdown (see Heilbrunn and Wilbur, 1937).

The inhibition of germinal-vesicle breakdown by prolonging the period of irradiation in sea water presents the interesting situation that the process producing germinal-vesicle breakdown can be inhibited even though sufficient calcium would seem to be present. Evidently, the succession of changes begun by the stimulating agent is governed by a rather delicate equilibrium. If the stimulus is of too short duration, the series of reactions may begin as indicated by a partial lifting of the membrane; but the process does not continue to the point of causing germinal-vesicle breakdown. If the irradiation is prolonged, again the series of reactions is begun, but germinal-vesicle breakdown is inhibited. Perhaps in this case an abundance of calcium inhibits the breakdown by inducing pronounced protoplasmic clotting.

SUMMARY

1. Sea-water–$MgCl_2$ mixtures reversibly inhibit germinal-vesicle breakdown produced by ultra-violet irradiation. The breakdown of the germinal vesicle can be inhibited by the addition of $MgCl_2$ subsequent to irradiation.

2. The inhibitory action of the $MgCl_2$ can be overcome by increasing the period of irradiation.

3. The action of $MgCl_2$ is antagonized by $CaCl_2$.

4. Germinal-vesicle breakdown and polar-body formation occur spontaneously in magnesium-free sea water.

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GROUP PROTECTION FOR EUPLANARIA DOROTOCEPHALA FROM ULTRA-VIOLET RADIATION

(One figure)

W. C. ALLEE AND JANET WILDER
University of Chicago

IN SOME of his earlier experiments Allee (1928) examined the effect of water in which planarians had been living and that in which other planarians had recently disintegrated on the ability of *Euplanaria dorotocephala* to withstand exposure to ultra-violet radiation. Under the conditions of those experiments such self-contaminated (conditioned) water hastened the onset and the progress of the cytolysis which is induced by ultra-violet irradiation of these worms.

The evidence at that time was recognized to be incomplete, since less contaminated water might have had a different effect. These experiments indicated that, when planarians were exposed to the action of ultra-violet rays when crowded together, retardation of cytolysis resulted or, with some worms, might be suppressed entirely. Such protective action was apparently a result of an interference with the penetration of the lethal rays, comparable with shading from visible light.

The present series of experiments have been conducted to explore further the effect of numbers present (*a*) during and (*b*) after exposure to ultra-violet radiation.

METHODS

The well-studied planarian, *E. dorotocephala*, was the only experimental animal used. All the worms were collected at a spring near Cary, Illinois. About 2,000 were placed together in large enamel pans and were maintained, until needed, under conditions which have been standardized by Professor C. M. Child during his long experience with planaria in this laboratory. The planarians were kept in water from a well in the Hull Biological Laboratory basement, and the worms were irradiated in this water except as noted elsewhere. Prior to use, the worms were starved for at least a week. They were then roughly divided into lots of 20 or more of approximately the same size and were measured as they crawled over co-ordinate paper. Those needed for a given experiment were usually counted out into finger bowls the day or night before they were to be treated, and were left on the water table near the stock containers until the next morning.

The experiments were begun in March, 1937, and continued to June, 1938.[1] Results of the treatment of more than 17,000 worms are reported here, not counting those in the nonirradiated controls. The worms averaged 13.6 mm. in length, and ranged from 6 to 20 mm. In any one series of an experiment the average variation in length was 2.5 mm.; the extreme variation was never more than 4.0 mm., and this occurred only with the largest worms.

[1] Additional tests of the influence of the H-ion concentration in the medium on survival after irradiation were made during the autumn of 1938.

All singly irradiated worms were exposed to ultra-violet rays in 5 cc. of water in Syracuse dishes; crowded worms (40–50) were exposed likewise. The treatment of the less crowded, so-called "grouped" worms varied. Usually 1 cc. of water was allowed for each of the grouped individuals. Thus there were 5 worms in 5 cc. in a Syracuse dish or 20 in 20 cc. in a Petri dish. However, in certain earlier experiments, in which the effects of well water were being tested along with other solutions, as many as 120 worms were irradiated together in a Petri dish in an attempt to reduce variability and so allow for a wider comparison of results. Regardless of the numbers exposed together, one invariable rule has been followed: all worms to be used in an experiment were given exactly similar irradiation and handling in so far as we knew how to do so.

The source of ultra-violet rays was a Cooper-Hewitt lamp, which was practically new at the start of these tests. It has a horizontal "Uviarc" tube and runs on a 110-volt direct current, using 4 amperes. The planarians were exposed to the full spectrum of this air-cooled mercury-vapor arc. For more uniform dosage from day to day, we allowed the lamp to warm up for about 20 minutes before usage.

The dishes which contained the worms to be irradiated were placed on an upturned crystallization dish which was covered by a black cloth. Prior to Experiment 8 the dish was given a quarter-turn approximately each 15 seconds; thereafter the same setup was used except that it was mounted on a motor-driven turntable which made about three revolutions per minute.

Exposure to the irradiation was measured in terms of time and distance. The vertical distance from the center of the arc to the center of the base on which the dishes rested varied from 21.3 to 26.5 cm. In later experiments it was standardized at about 25.5 cm. Exposure varied from 2 to 10 minutes; it was usually from 4 to 6 minutes and was planned to bring on definitive lesions in from 1 to 3 hours.

Under the stimulus of the irradiation the worms writhe and twist; these reactions tend to result in their piling-up in the angles around the margin of the dishes. In order to insure a full exposure of each worm, one of us stirred the worms back toward the center, using a camel's-hair brush, and scattered incipient clumps.

Before starting to irradiate the worms, from four to eight series of the saltcellar type of embryological dishes were arranged near by; each series contained eleven dishes, one for the group and ten for the isolated animals. In Experiments 1–38 each contained approximately 8 cc. of the water into which the worms were to be placed. Beginning with Experiment 38, each contained a measured 5 cc. of this water.

The worms were transferred as soon as possible after irradiation into this fresh untreated water. A camel's-hair brush was used for the transfer. When various sorts of water were tested in a single experiment, a different brush was used for each type of water; and when conditions called for it, the worms were rinsed through a bath of the water in which they were to be observed. Ten irradiated worms were selected at random and placed together in one of the saltcellars; 10 more worms, which had been irradiated in the same dish (or dishes), were isolated into ten similar dishes; and so on for the various series. It is worth emphasizing that the worms were never assayed in the water in which they had been irradiated.

At all times in the following accounts, worms said to be "grouped" (G) under the ultra-violet lamp had been exposed in a Petri dish, usually 20 worms in 20 cc. of water, about 4.5 mm. deep. Those worms said to be grouped during the survival period had been placed in lots of 10 in a saltcellar embryological dish. When worms are said to be

"crowded" (Cr), they were present in such numbers during irradiation that they shade each other. All isolated worms (S) were placed 1 to each container.

During the period of assay, from time to time the worms were inspected under a hand lens or binocular dissecting microscope. The intervals between inspections depended on how rapidly disintegration was proceeding. At times it was necessary, particularly in the early stages following irradiation, to move the worms somewhat to allow critical inspection. This was accomplished with the aid of a series of glass rods, one for each type of water in the experiment. The effect of even more severe agitation was tested in Experiments 78–91, and we believe this slight disturbance had no appreciable differential effect on the time before onset of cytolysis. Frequently, but not always, the same number of isolated worms were disturbed by the glass rod that had been touched in the group, or vice versa. This careful equalization of disturbance did not seem to change the experimental results when compared with the more casual method of touching only those worms which were otherwise difficult to inspect.

Records were kept on appropriately mimeographed sheets. A system of arbitrary designation of stages was evolved early, as follows:

Stage 0. No effect to be observed.
Stage 1. Worm only generally affected; clings tightly to the substratum and appears drawn together.
Stage 2. Local effects but no lesions; margins may be rolled and swollen, usually anteriorly.
Stage 3. First lesion; usually at the anterior tip but may occur at the posterior tip, rarely in the mid-dorsal region. (If the exposure has been light, this stage may be transitory and the worm may return to an earlier stage for the time being.)
Stage 4. First lesion extends to pharynx or a second appears.
Stage 5. All dorsal integument cytolyzed.
Stage 6. Only a fluffy mass remains.

These stages are more or less arbitrary and represent the more easily recognized divisions of a continuous process. No attempt has been made to determine the physiological value of the intervals between the different stages. The step from stage 2 to stage 3 may have, for all we know, a different significance than that between stages 3 and 4.

The records for each set of 10 planarians, whether isolated or grouped, were added. A total stage of 30 for ten worms (that is, a mean stage of 3) was taken as the criterion for determining survival. This was done because stages 2, 3, and 4 are most clearly defined and enter chiefly into this total. Total stage 40 was almost always recorded as a check. However, if this was not reached within 12 hours, it could not be used, since experiments rarely continued longer. The records for "40" normally support the results found for "30," and little use will be made of them now beyond noting this fact (see Table 1).

The worms were treated throughout at room temperature, which ranged from 19°.4 to 28° C. during the whole series of experiments. The mean temperature was 22°.7 C., and this approaches the more usual conditions. Control series were used in the first twenty-three experiments. Through Experiment 15 and again near the end of the experimentation, these animals were irradiated *under glass*, along with those to be assayed. Later they were not irradiated but were placed in a suitable control series near the worms that had been exposed to ultra-violet rays. Neither type of control showed any cytolysis in the course of an experiment. Having established the fact that, aside from the ultra-

violet irradiation, the experimental conditions had no action, nonirradiated control series were discontinued except when room temperature was abnormal or when the worms were assayed in distilled water or some other unusual medium.

EXPERIMENTAL RESULTS

The majority of the data will be presented in the form of highly condensed summarizing tables. The amount of variation will be indicated to the initiated by the statistical probability of securing the same differences as a result of random sampling, which will be given as *P*. Probabilities have been calculated by the use of "Student's" method for

TABLE 1

A SAMPLE EXPERIMENT (NO. 13) SHOWING CYTOLYSIS STAGES OF THE INDIVIDUAL WORMS IN THE FIVE SERIES OF WHICH IT CONSISTS

Each series is considered alone; the 10 members of the group are compared with the 10 single animals, at the assay "reading" at which the singles are nearest to total stage 30. "G" grouped and "S" isolated animals. For stage values see text, page 112.

Animal No.	Series A		Series B		Series C		Series D		Series E	
	G	S	G	S	G	S	G	S	G	S
1	2	3	1	3	1	3	1	3	1	3
2	3	3	1	3	1	3	2	4	3	3
3	3	3	2	3	1	3	2	3	3	3
4	3	3	2	3	1	3	2	3	3	3
5	3	3	2	3	1	3	2	3	3	3
6	3	3	3	3	2	3	3	3	3	4
7	3	3	3	3	2	3	3	3	3	4
8	3	3	3	3	3	3	3	3	3	3
9	3	3	3	3	3	3	3	3	3	2
10	3	3	3	3	3	3	3	3	3	3
Total stage value	29	30	23	30	18	30	24	31	28	31
Minutes from start	52	52	33	33	41	41	55	55	43	43
Minutes to "30"	61	52	123	33	137	41	107	41	78	39
Minutes to "40"	155	122	221	153	259	201	190	150	153	121

data from paired experiments. When *P* equals or is less than 0.05 (five chances in a hundred of finding a like result in random sampling), the results should be given serious attention, the more so the smaller the fraction.

For many, such statistical expressions still lack meaning; hence, at the beginning we submit, in Table 1, the data from one experiment which we trust is fairly typical. Each line in all of the following tables is a summary of such data. The number of paired comparisons which are summarized is given in each case; to obtain the number of worms used, one can usually multiply this number by 20.

The effect of numbers present during irradiation.—Certain of the effects of the degree of crowding during irradiation are summarized in Table 2. In the column marked "Comparisons" the first symbol in each pair indicates the numbers present during irradiation; the second symbol shows the condition during the assay period. Thus, the first entry, CrS:GS, means that a lot of worms irradiated as a crowd and assayed while

isolated are compared with others which were irradiated as a group and likewise assayed singly. The other items in the table have been explained in the section, "Methods." The observed survival to total stage "30" in minutes is given in the fourth column, and the relative survival in percentages of time is shown in the seventh column. Statistical probabilities (*P*) are calculated for both.

In the first four sets of comparisons the worms were assayed while isolated; in the last three they were grouped. The effect of numbers present while being irradiated shows more clearly under the former condition. There is much variation in the survival of worms from the different experiments—so much so that cross comparisons are not practical. These differences result from the large number of known variables from one experiment to the next, some of which are size, condition of the planarians, time of exposure, distance from the lamp, and temperature.

TABLE 2

THE SURVIVAL OF PLANARIANS WHICH HAD BEEN IRRADIATED AS "CROWDS" OR GROUPS OR ISOLATED, AND THEN ASSAYED ALL ISOLATED (UPPER HALF OF TABLE) OR ALL GROUPED (LOWER HALF) DURING CYTOLYSIS

Comparison*	No. of Paired Comparisons	Total No. of Worms	Minutes to "30"	Difference	*P*	Percentage	Difference	*P*
CrS:GS.....	6	120	517:119	398	0.132	100:54	46	0.079
GS:SS.....	7	140	107:40	67	0.092	100:60	40	0.031
CrS:SS.....	6	120	517:41	476	0.090	100:24	76	0.0001
GS:SS.....	23	460	168:140	28	0.031	100:86	14	0.010
CrG:GG.....	6	120	723:172	551	0.079	100:51	49	0.132
GG:SG.....	4	80	189:80	109	0.208	100:62	38	0.105
CrG:SG.....	4	80	907:80	827	0.139	100:32	68	0.068

*"Cr," "G," and "S" as prefixes indicate irradiation in crowds, groups, and isolated, respectively. "G" and "S" suffixes show that after irradiation the worms were grouped or isolated, respectively, during cytolysis.

The variation in survival is especially high when the worms are irradiated in the crowded condition. For example, some of the worms, more than others, may remain at the top and hence get a different exposure. With "grouped" worms this type of direct protection is largely avoided, and it is impossible for those which are irradiated singly.

As a result of this variation, despite wide differences in the mean survival time in minutes, only one of the comparisons is significant when difference in minutes before onset of cytolysis ("30") is the criterion for comparison. The one significant difference (GS:SS) has the smallest mean difference of all. It is, however, the result of the largest number of comparisons, and this suggests that with more data some of the others might be significant. In cases like these it is frequently more fair to consider the difference on a percentage basis. When this is done, all the lots irradiated and assayed as isolated worms give significant differences. The apparent discrepancy between mean percentage difference and mean time difference is on account of the fact that percentage has been calculated case by case, with the longer survival evaluated as 100 per cent. If there were exceptions to the general trend, the mean percentage on the side which tends to survive longer will not be 100. For convenience in such cases this value has

been raised to 100, and the lower value on the opposite side of the compared series has been comparably corrected to maintain the same proportional mean deviation.

In common English this table shows that, when assayed singly, densely or even slightly crowded worms while being irradiated, survive better than if irradiated singly. The crowding effect was reported earlier (Allee, 1928) and is to be expected. The protection which the uncrowded group furnishes is not easily explained. What effects do these worms have on each other, either directly or by change in the water about them, that permit them to live longer after irradiation than do other worms which have been exposed similarly in all respects except that they were isolated? The fact that the grouped worms are protected is plain. The explanation, however, is not obvious. It must be remembered in this connection that these worms were not allowed to pile up during irradiation; hence the easiest explanation is untenable. Since they were assayed in fresh, nonirradiated water, mass protection from some effect of irradiation on the water—the production of ozone, for example—is not operating.

When assayed in groups, that is, with 10 worms in 5–8 cc. of water, differential effects of numbers present during irradiation are not significant. The mean differences in time to total stage 30 are even greater than before, but the variations within an experiment and from experiment to experiment are also much greater. If the trends were to continue as in these tests, further experimentation might be expected to yield significant data. Such a possibility, while interesting, does not affect the fact that with the tests as made, given equivalent differences in crowding during irradiation, significant differences are shown by the assay of isolated worms which are not revealed with the grouped planarians. This statement holds even though we do not consider the results given in the second test of the GS:SS relations, and hence limit ourselves to those which were nearly equivalent as far as numbers of worms are concerned.

The isolated state provides a more sensitive method of discovering the effects during and after irradiation than does the grouped condition. When isolated, the cytolysis of a worm affects itself only; when grouped with others in the same medium, all or a part of the activities of any individual worm preliminary to, or during, cytolysis may affect others, as well as the worm itself.

The effect of numbers present after irradiation.—In order to obtain data on the importance of numbers present during the period of assay after exposure to ultra-violet rays, all the worms being compared in any one test must be given an equal exposure. They must all be irradiated when crowded or grouped or isolated. Our experience showed more uniform results with worms irradiated as groups when the precautions previously stated were taken than when the worms were crowded together. Such group irradiation is more economical of time and perhaps more uniform than is that of the isolated worms; hence, most of our data were obtained from worms which had been exposed in groups. The results are summarized in Table 3.

We have collected much evidence on this point. These comparisons are labeled GG:GS in Table 3. The first set of tests was made in the spring of 1937. These were repeated in the following autumn to find if the same conditions held at that season. Four other repetitions were made as controls for tests of other factors. In each of the series the mean survival of planarians, equally exposed to ultra-violet, was longer if 10 worms were present together after transfer to fresh well water than if a similar number of worms were isolated each into the same amount of water which was occupied by the group. The fact that similar results have been obtained in so many different repeti-

tions in which large numbers of planarians were used in each series, combined with the relative uniformity of the percentage differences and the high statistical significance, is strong evidence of the reality of the observed difference. Enough tests were made of worms exposed singly and then assayed for survival, half grouped and half still isolated (SG:SS), to show that the group protection is present under these conditions. The variation in the exposure of individuals when they are crowded together (Cr), which has

TABLE 3

THE EFFECT OF NUMBERS PRESENT AFTER IRRADIATION UPON CYTOLYSIS

Comparison*	No. of Paired Comparisons	Total No. of Worms	Minutes to "30"	Difference	*P*	Percentage	Difference	*P*
GG:GS.....	64	1,280	148:78	70	0.021	100:78	22	0.0001
GG:GS.....	23	460	247:168	79	0.0001	100:73	27	0.0001
GG:GS.....	12	240	184:144	40	0.024	100:82	18	0.007
GG:GS.....	12	240	135:110	25	0.003	100.84	16	0.0008
GG:GS	19	380	64:50	14	0.005	100:82	18	0.006
GG:GS.....	25	500	169:123	46	0.038	100:86	14	0.0012
SG:SS.....	4	80	80:32	48	0.208	100:52	48	0.049
CrG:CrS.....	6	120	723:517	206	0.054	100:74	26	0.132

* In each pair of symbols the prefix shows the condition during irradiation and the suffix during assay. As before, "S" means isolated, "G" grouped, and "Cr" crowded. These symbols are used throughout and will not be explained again.

TABLE 4

THE SURVIVAL OF WORMS GROUPED OR ISOLATED THROUGHOUT THE PERIOD OF IRRADIATION AND OF ASSAY

Comparison	No. of Paired Comparisons	Total No. of Worms	Minutes to "30"	Difference	*P*	Percentage	Difference	*P*
GG:SS.....	7	140	158:40	118	0.089	100:40	60	0.0001
GG:SS.....	23	460	247:140	107	0.0001	100:60	40	0.0001

been mentioned before, accounts for the failure of worms so irradiated to show a very significant difference in survival when grouped and isolated during the period of assay; and this despite the mean difference of 206 minutes before the onset of cytolysis.

Differences in numbers present during both irradiation and assay.—The preceding tables and discussion have shown clearly our experience that, by some mechanism, grouping either during irradiation or during the period that follows before cytolysis, or during both periods, favors the survival of these planarians. As might be expected, when worms were assayed which had been grouped when irradiated and during the later period of observation and compared with those that had been isolated during both experiences, the grouped worms were found to survive decidedly longer. The pertinent experimental data have been summarized in Table 4.

Since such results are merely a verification of the work already presented, there is no call for extended discussion. All that needs to be stated is that the comparisons were, so far as we know, fairly made. The isolated worms were exposed under the same conditions (including depth of water) as were those which were grouped. The different sets of grouped and isolated worms were irradiated simultaneously in lots of 5 grouped and 5 isolated worms. Again, as always, all worms were transferred to fresh water immediately after irradiation. Table 4 shows that the observed differences are highly significant.

Groups versus isolated worms in other waters.—Well water from the Hull Laboratory well is rich in calcium and has a pH of 7.7 or more. The water from a somewhat similar

TABLE 5

SURVIVAL OF GROUPED AND ISOLATED WORMS IN SYNTHETIC POND WATER (*A*) AND IN DOUBLE-DISTILLED WATER (*B*)

Comparison*	No. of Paired Comparisons	Total No. of Worms	Minutes to "30"	Difference	*P*	Percentage	Difference	*P*	pH
				A					
GG:GS.....	11	220	126:120	6	0.562	100:99	1	0.854	6.8
RGG:RGS...	18	360	259:261	2	0.922	100:100	0	1.000	6.8
GG:GS.....	8	160	284:184	100	0.055	100:69	31	0.035	7.7
				B					
RGG:RGS...	14	280	267:137	130	0.004	100:64	36	0.0004	.....
RGG:RGS...	8	160	49:39	10	0.023	100:82	18	0.011	.....
RGG:RGS...	7	140	46:31	15	0.140	100:75	25	0.053	.....
RGG:RGS...	4	80	43:41	2	0.591	100:94	6	0.591	.....
RGG:RGS...	9	180	454:179	275	0.110	100:69	31	0.050	.....

* Symbols as in preceding tables except that here "R" shows that irradiation took place in the same type of water as that in which the worms were assayed.

well in the Whitman laboratories, which differs in being near relatively large animal colonies that may contaminate the ground water, is known to vary in chemical composition from time to time. In an effort to secure reproducible water, we have accordingly developed a simple synthetic pond water made by adding "reagent quality" salts to water distilled first in an aluminum still and usually redistilled in glass. Whenever distilled water is mentioned in the following pages, this double-distilled water is meant. The resulting "synthetic pond water" contained, per liter, 0.1 gm. $CaCl_2$ and 0.05 gm. each of $MgSO_4$, K_2SO_4, and $NaNO_3$. The pH of this solution was between 6.0 and 7.0, depending on the length of time it had been exposed to the air. For some experiments this was raised to 7.7 by the addition of NaOH. Results of tests made in this water and in double-distilled water are given in Table 5.

The planarians marked GG:GS in this table were irradiated in well water and transferred to the synthetic pond water for assay. Those planarians having these

symbols preceded by "R" were irradiated in the same type of water in which they were assayed and then were transferred to fresh water of that type to find the length of their survival. Apparently it made no difference in the group-single differential in survival whether the worms were irradiated in well water or in synthetic pond water. In both sets of experiments, results from which are summarized in the first two lines of Table 5, *A*, there was no significant difference in survival under the conditions tested. When, however, the pH was adjusted to that of well water (7.7), the isolated worms again cytolized more rapidly than their accompanying groups, as they did also in the similar tests which were made simultaneously in well water. Some aspects of the significance of this observation will be discussed in a later section.

If one were to focus solely on the data summarized in the first line of Table 5, *B*, it would appear certain that the group-single differential in survival after irradiation also holds when the worms are in distilled water. Later tests, some of them with decidedly smaller numbers, were not so decisive. The grouped worms averaged a longer survival in each case, but only in one other set of comparisons are the results strikingly significant. When the whole five sets of experiments are considered together on the percentage basis, groups survived, on the average, 23 per cent longer than the accompanying isolated worms. This difference has a statistical probability of 0.0116 and must be taken seriously. As stated before, when there are so many known variables between different sets of experiments, it is fair to use the percentage difference as a basis of comparisons.

During the time taken to complete an experiment, control lots of nonirradiated worms in similar distilled water were completely unaffected, so far as cytolysis was concerned. The irradiated planarians in distilled water were much more susceptible to the treatment with ultra-violet than were worms simultaneously assayed in well water. In fact, with one dosage used (4 minutes at 26 cm.) the latter were unaffected.

There seems to be no room for doubt but that under a variety of conditions grouped worms which have been given a lethal exposure to ultra-violet irradiation survive longer than do accompanying isolated worms which have similarly and simultaneously received the same ultra-violet dosage. We are now ready to undertake an analysis of the factors underlying such group protection.

The effect of "conditioned" water.—During assay the planarians usually lived for some hours in the few cubic centimeters of water in the observation dishes. During this time the worms give off certain substances to the water, among which in distilled water are known to be: mucus; electrolytes, including calcium; carbon dioxide; and in later stages of cytolysis, proteins. Bacteria doubtless break down the organic material into decomposition products which are at present unknown. The alterations in water as a result of such living and disintegration we call "conditioning." This process will obviously proceed faster when 10 worms are placed together than if 1 worm is isolated into the same amount of water.

For an analysis of the effects of such conditioning we placed an irradiated worm in 2 cc. of water in one of the usual saltcellars and immediately or later added the irradiated worm whose survival was being followed. The two were selected so that they could easily be distinguished. If the "conditioning" worm had been lightly irradiated and was intact during most of the period of assay, the water was said to be lightly conditioned. If it was or soon became completely cytolyzed, the resulting water was regarded as being heavily conditioned.

In thirteen paired cases involving 260 worms in well water, the worms in lightly con-

ditioned water survived an average of 45 minutes longer than did similarly treated ones in heavily conditioned water ($P = 0.005$). On the usual percentage basis this amounts to a mean difference of 19 per cent ($P = 0.005$). This finding that the worms survived a shorter time in heavily conditioned water is in keeping with the results reported by Allee (1928). There was, however, no significant difference to be found after using seventeen series (340 worms) between those isolated into 2 cc. of fresh well water and those in lightly conditioned well water; or between fourteen series (280 worms) in which planarians were isolated, on the one hand, into 5 cc. of fresh well water and, on the other hand, into 2 cc. of such water.

These tests were repeated in distilled water without obtaining any significant differences. Evidently, in these exploratory experiments we did not reproduce the conditions that are obtained when 10 irradiated worms are placed together in 5 cc. of water.

TABLE 6

ANALYSES OF TIME TO REACH TOTAL STAGE 30 FOR PLANARIANS IRRADIATED IN WELL WATER AND ASSAYED IN PLAIN WELL WATER AND IN SUCH WATER WITH CALCIUM ADDED

(Experiments Involve a Total of 770 Animals)

Comparison*	No. of Paired Comparisons	Minutes to "30"	Difference	*P*	Percentage	Difference	*P*
Ca:W.....	17	66:56	10	0.008	100:88	12	0.035
WGG:WGS.....	19	64:50	14	0.005	100:82	18	0.006
CaGG:CaGS.....	19	70:60	10	0.001	100:88	12	0.009
CaGS:WGS.....	18	60:49	11	0.012	100:85	15	0.034
CaGG:WGG.....	18	70:63	7	0.152	100:91	9	0.128
CaGG:WGS.....	18	70:49	21	0.0004	100:74	26	0.0006
WGG:CaGS.....	18	63:60	3	0.381	100:98	2	0.694

*"W," well water; "Ca," well water with increased calcium.

The effect of calcium.—One of the decided differences between distilled and well water is the absence of calcium from the former. Earlier work (Oesting and Allee, 1935) had shown that calcium retards cytolysis of marine *Procerodes* placed in fresh water and of *Euplanaria* when in distilled water (Buchanan, 1935). This similar deficiency of calcium may be the reason why the planarians in distilled water survived the effects of ultraviolet irradiation for so much shorter a time than did those simultaneously and equally exposed and assayed in well water.

Well water with increased calcium: The well water which was the basic medium for much of this work contained between 40 and 50 mg. of calcium per liter. Enough $CaCl_2$ was added to make a M/120 solution in well water. The planarian worms were irradiated in well water and then for the assay period were transferred to fresh well water or to the water with calcium added. Results are summarized in Table 6.

As one reads down Table 6, it is evident that in the seventeen cases in which comparisons are entirely fair, the grouped and isolated worms, considered together, survived 10 minutes longer ($P = 0.008$) in well water to which calcium had been added than they did in well water alone. In both media the usual group-single differential showed

significant differences. The isolated worms lived longer in the stronger calcium solution, while there was an insignificant difference with grouped worms. This verifies findings already reported that the assays made on isolated worms are more sensitive than those made with grouped planarians.

In keeping with all these results, the grouped worms in the well water plus calcium survived better than did the isolated ones in well water. However, the added protection furnished the group in well water caused such worms to live slightly but insignificantly longer than did isolated individuals in the calcium-rich water.

TABLE 7

SHOWING SURVIVAL AFTER IRRADIATION WHEN ASSAYED IN SEVERAL DILUTIONS OF $CACL_2$

(Specific Conductivity in Mhos $\times 10^{-6}$)

Dilution	Comparison	No. of Paired Comparisons	Minutes to "30"	Difference	*P*	Percentage	Difference	*P*	Specific Conductivity
M 0.0069....	WGG:WGS	8	49:39	10	0.023	100:82	18	0.011	1.6
	CaGG:CaGS	8	386:201	185	0.047	100:68	32	0.020	2,209
	CaGG:WGG	8	386:49	337	0.009	100:21	79	0.0001	.....
	CaGS:WGS	8	201:39	162	0.003	100:23	77	0.0001	.....
	Ca:W	8	294:44	250	0.005	100:30	70	0.0001	.....
M 0.001.....	WGG:WGS	7	46:31	15	0.140	100:75	25	0.053	.....
	CaGG:CaGS	12	445:386	59	0.162	100:71	29	0.007	252.3
	CaGG:WGG	9	442:39	403	0.027	100:12	88	0.015	.....
	CaGS:WGS	8	497:29	468	0.041	100:11	89	0.0001	.....
	Ca:W	7	526:38	488	0.061	100:13	87	0.0001	.....
M 0.00001...	WGG:WGS	4	43:41	2	0.591	100:94	6	0.591	.....
	CaGG:CaGS	4	103:87	16	0.127	100:80	20	0.068	4.5
	CaGG:WGG	4	103:43	60	0.115	100:50	50	0.014	.....
	CaGS:WGS	4	87:41	46	0.208	100:59	41	0.032	.....
	Ca:W	4	95:42	53	0.154	100:53	47	0.012	.....
M 0.000001..	WGG:WGS	9	454:179	275	0.110	100:69	31	0.050	.....
	CaGG:CaGS	9	697:250	447	0.007	100:39	61	0.0001	1.5*
	CaGG:WGG	9	697:454	244	0.047	100:56	44	0.005	.....
	CaGS:WGS	9	250:179	71	0.148	100:79	21	0.007	.....
	Ca:W	9	474:317	157	0.015	100:60	40	0.002	.....

* Distilled water at this time tested 1.4×10^{-6} mhos.

Such results showing the importance of calcium in survival of irradiated planarians led to an attempt to find the minimum amount which could be added to secure protection. Tests were made in solutions of $CaCl_2$ in distilled water of the following strengths: 0.0069 M, 0.001 M, 0.00001 M, and 0.000001 M. All exposures to ultra-violet radiation were made in double-distilled water. Irradiated controls were tested simultaneously in the distilled water, and nonirradiated controls were also used. These latter showed some lesions in the weaker dilutions but never approximated the rate of cytolysis of the animals treated with ultra-violet. Summaries of these tests are given in Table 7.

For reasons already noted, when the known variables are as many and as large as in

these experiments, the percentage comparisons are fairer than a study of time differences. Focusing, then, on the percentage columns, one notes that the group versus isolated comparisons in distilled water do not always yield significant results in each separate series; when, however, the whole experience with distilled water is considered together, there is no doubt but that the worms assayed in groups live longer after exposure to ultra-violet than if they are isolated.

Similar comparisons in the various dilutions of $CaCl_2$ also show significantly longer survival if assayed in groups than if isolated. All but one of the series tested is clearly significant, and there is no doubt of the difference when all the series are considered together. In all comparisons, worms assayed in various concentrations of $CaCl_2$ solutions survived longer than did similar worms in distilled water. This holds true even with group comparisons, which are less critical in many ways than are those of isolated worms. As would be expected, with both separate parts significant, the combined comparisons are highly so.

It is worth noting that a solution of M 0.000001 $CaCl_2$ with a conductivity of only 1.5×10^{-6} mhos still gave decidedly longer survival after irradiation than did distilled water with a slightly less conductivity (1.4×10^{-6}). Buchanan (1935) found that similarly extreme dilutions of calcium protected *E. dorotocephala* when placed in hypotonic water without having been irradiated. In our experience, after irradiation cytolysis was delayed by exposure either to hypotonic or to hypertonic solutions of $CaCl_2$.

Parallel experiments with similar concentrations of $MgCl_2$ demonstrated that this salt also delays cytolysis after irradiation with ultra-violet; the effect, however, is not so marked as with similar concentrations of $CaCl_2$. This relationship can be stated without giving even the abbreviated summaries we have been using. Both M 0.001 and M 0.00001 solutions of $CaCl_2$ delayed cytolysis significantly longer than did the same strength of $MgCl_2$. With M 0.000001 the worms survived 21 per cent longer in $MgCl_2$ than in distilled water ($P = 0.128$), while they survived 40 per cent longer in $CaCl_2$ ($P = 0.002$).

Other experiments verified these results. With artificial pond water it is possible to use $MgCl_2$ in place of $CaCl_2$. The solution then contained, per liter of distilled water, 0.1 gm. $MgCl_2$, 0.05 gm. each of $MgSO_4$, K_2SO_4 and $NaNO_3$. At times worms were entirely normal in appearance after 4.5 days in such water, and in no case was a nonirradiated control in this calcium-free water affected during the progress of an experiment. After irradiation grouped and isolated worms survived significantly longer in well water and in the regular synthetic pond water than they did in calcium-free synthetic pond water.

These observations, especially the results with M 0.000001 $CaCl_2$, suggest that, if the group gives off more calcium into the assay water than do the isolated worms, this may be the mechanism of the group-single differential.

Proportional volumes of well water.—In order to test the effect of proportional, rather than equal, volumes on survival after irradiation, the planarians, exposed in lots of 20 per Petri dish in well water as usual, were then placed in saltcellar embryological dishes, 10 together in 10 cc. of fresh well water and 1 each in ten dishes in 1 cc. of similar water. The volume of water was thus proportional to the number of worms being tested; the depth was unequal, but the exposed surface was approximately the same for grouped and for each isolated worm. To avoid excessive evaporation, all were kept in moist chambers. The results are summarized in Table 8, *A*. With the volume of water during assay proportional to the number of worms present and arranged, so that there was 1 cc. of water

for each worm whether grouped or isolated, the worms still lived significantly longer if assayed when grouped. The difference in minutes is 178 with $P = 0.019$.

As a further refinement, after irradiation as before, 9 worms were put into the usual embryological dishes in 9 cc. of water, and 9 others were isolated in 1 cc. of water, each in a narrow shell vial. The depths were practically the same, although the exposed surface was much less in the shell vials. Under these conditions, as shown in Table 8, *B*, the usual group-single differential was destroyed; and while there was no significant difference, the isolated animals averaged longer survival.

These results, taken together, indicate that in the tests as usually made, where volume, surface area, and depth were all equal regardless of numbers present, there may have been some volatile substance (carbon dioxide, for example) which accumulated more rapidly among the grouped worms and retarded their cytolysis.

TABLE 8

SURVIVAL IN PROPORTIONAL VOLUMES OF WATER: A, WITH UNEQUAL DEPTH AND EQUAL SURFACE; B, WITH DEPTH EQUAL AND SURFACE UNEQUAL

(*A*, 340 Worms; *B*, 280 Worms)

Comparison	No. of Paired Comparisons	Minutes to "30"	Difference	*P*	Percentage	Difference	*P*
	A						
GG:GS.....	17	474:296	178	0.019	100:68	32	0.0002
	B						
GG:GS.....	14	218:237	19	0.336	95:100	5	0.496

The effect of agitation on cytolysis.—During inspection of the planarians for possible effects of irradiation, it was sometimes necessary to move the worm slightly in order to secure adequate inspection. This was more likely to be true for grouped worms. For this purpose we used a rounded glass rod and handled the worms as gently as possible. Sometimes when one or more worms of the grouped lot were touched, a similar number of the accompanying isolated series would be similarly treated. Often this was not done. It is possible that such mechanical stimulation may have hastened the onset and progress of cytolysis. If so, the grouped worms should have been more affected, and this would have worked against the observed results.

In order to test the effectiveness of this factor, we set up parallel series of worms each with the usual groups of 10 and 10 accompanying isolated individuals. One such set was left undisturbed until near "30," and then the reading was taken with the minimum disturbance. In another series all animals were briskly stirred with a camel's-hair brush about five times between each reading until "30" was reached. An analysis of the results obtained appears in Table 9.

As usual, the group-single differential survival shows in the unagitated worms, while

in the agitated ones it has entirely disappeared. The situation, however, is not quite this simple, for the disturbed groups survived a significantly shorter time than did those which were undisturbed. The isolated worms, on the other hand, show no significant difference. When averaged together, the agitated grouped and isolated worms survived a significantly shorter time than the unagitated ones.

The fact that the isolated worms are not significantly affected by the disturbance given them indicates that the results are not caused by direct action of the agitation on the worms themselves. In the usual assays any disturbance of the worms would tend to break down the observed group-single differential survival; and since, in actual practice, group worms were touched more than isolated ones, the observed group effect, as demonstrated, for example, in Table 3, cannot have been a result of differential stirring while making the observations.

This evidence indicates that the agitation may disperse some material from the water around the grouped worms which otherwise would delay cytolysis. Calcium would not

TABLE 9

THE EFFECT OF AGITATION ON RATE OF CYTOLYSIS

(Each Comparison Involves 12 Paired Cases and a Total of 240 Worms)

Comparison*		Minutes to "30"	Difference	*P*	Percentage	Difference	*P*
Unag.	GG:GS	135:110	25	0.003	100:84	16	0.001
Ag.	GG:GS	101:101	0	1.000	99:100	1	0.922
Groups	Unag:Ag	135:101	34	0.005	100:77	23	0.002
Isolated	Unag:Ag	110:101	9	0.162	100:91	9	0.099
Combined	Unag:Ag	123:101	22	0.012	100:83	17	0.005

*"Ag," agitated; "Unag.," unagitated.

be much affected by such treatment, while carbon dioxide would escape readily from the agitated water.

The effect of pH on rate of cytolysis.—The experiments here reported grew out of the observation that the survival of worms in regular synthetic pond water was much longer than in well water. Among the known variants are: The synthetic pond water is an unbuffered water with approximately half the calcium content found in well water. Also, the pH of the well water is much higher, ranging from 7.7 to 8.2. The synthetic pond water, on the other hand, has a pH comparable with distilled water; in the spring of 1938 both were about pH 6.0 when fresh and approached neutrality on standing.

The pH was changed, as needed, by adding a few drops of 0.1N NaOH or HCl. Carbonate-free well water was obtained by acidifying well water with concentrated HCl below pH 4 and then allowing a stream of compressed air to bubble through for about 18 hours; after this the pH was raised to the desired level with NaOH. Hellige indicator and standards were used to determine pH.

The treatment of animals and the experimental setup were as usual unless mentioned otherwise. The worms were irradiated in synthetic pond water or in well water, depending on which was basic for the given experiment. After rinsing with fresh water of the type to be used in the assay, they were transferred to the assay dishes. With three exceptions (tests 1, 2, and 5 of Table 10, *B*) all animals were assayed singly in series of 10

isolated worms. In the case of the exceptions, groups and singles have been considered together. At the time of filling the assay dishes, one extra dish for each medium was also filled with 5 cc. of that water, and 0.5 cc. of Hellige indicator was added. In this way the pH, together with any drift, could be read at any time during the experiment provided the light was suitable.

In the experiments made during the autumn of 1938 the pH of unbuffered waters shifted slightly, if at all. Only acidified well water displayed this phenomenon to any

TABLE 10

THE EFFECT OF pH ON SURVIVAL AFTER IRRADIATION

(Specific Conductance Is Given in Mhos×10^{-4})

Water 1:2	Mean pH 1:2	No. of Paired* Comparisons	Minutes to "30"	Difference	*P*	Percentage	Difference	*P*	Specific Conductance† 1:2
	A, Comparisons within One Medium between pH below and above 7.0								
Well:Well	5.8:7.6	3	194:127	67	0.105	100:68	32	0.039	3.3:2.8
	6.3:8.0	8	309:162	147	0.013	100:54	46	0.001	3.2:2.8
SP:SP	6.2:8.3	12	169:195	26	0.295	95:100	5	0.498	.....
	6.7:8.0	12	166:165	1	0.922	100:99	1	0.627	2.9:2.9
CO_3-free:CO_3-free	6.2:7.8	11	136:175	39	0.037	85:100	15	0.009	equal
	B, Comparisons between Two Media of Different or Same pH								
SP:Well	6.8−:7.5+	6	377:110	267	0.017	100:35	65	0.000	.....
	6.8−:7.5+	11	124:86	38	0.001	100:72	28	0.031	.....
	6.7:8.0	4	134:80	54	0.068	100:63	37	0.012	2.9:2.8
	6.4:6.5	18	144:178	34	0.061	88:100	12	0.009	2.9:3.2
CO_3-free:Well	6.2:7.7	5	229:146	83	0.027	100:66	34	0.023	6.9:2.8
	6.3:6.1	6	218:150	68	0.021	100:70	30	0.004	6.8:3.3
	6.3:6.3	7	167:182	15	0.047	91:100	9	0.041	8.0:8.8
	5.8:5.7	12	156:147	9	0.295	100:98	2	0.628	9.0:8.9

* To find the number of worms used in each test, multiply the number of paired comparisons by 20, except in the first, second, and fifth test under *B*, where 40 worms have been used in each paired comparison.

† Specific conductance has been deduced for most of these waters by tests on similar water of similar pH. In the last three tests, however, the specific conductance is that of the same lot of water used in the survival assay.

marked extent. (There is evidence of reduced ammonia fumes in the air, as compared with the spring of 1938.) Acidified well water showed a shift in pH of 0.1 unit per 14 or 15 minutes. Unmodified well water, on standing, had a slow pH drift up to pH 8.3 at the rate of 0.1 unit per 34–60 minutes. Carbonate-free well water initially below pH 6.4 showed a mean rise at the rate of 0.1 unit per 75 minutes (extremes 17.5 to no rise at all). Synthetic pond water of low pH gave irregular results; it showed a rise, if at all, of 0.1 unit in an average of 113 minutes (extremes, 18 minutes to no change in 5 hours).

Our experience regarding the effect of pH on survival after irradiation is summarized in Table 10. The experimental results indicate:

1. Isolated irradiated planarians survive significantly longer in well water below

neutrality. If, however, the carbonates are first removed from this water, they then survive significantly longer in water above pH 7.0.

2. In synthetic pond water (which lacks carbonates) there is no difference in the survival of the isolated worms in the water above and below pH 7.0.

3. The planarians survive significantly longer in synthetic pond water at its usual pH than in unmodified well water, which usually has a pH approximately a whole unit higher.

4. When, however, the well water is acidified below neutrality, survival of the worms is increased over that occurring in synthetic pond water.

5. Animals tested in carbonate-free well water and in well water of low pH survive significantly longer in the former. This is, however, readily explained by the large increase in the electrolytic content of the carbonate-free water. After adjustment of this factor is made, it is found that—

6. When the worms were tested in carbonate-free well water, as contrasted with ordinary well water brought to the same pH, and in which the electrolytic content slightly exceeded that in the carbonate-free well water, the opposite condition obtained; i.e., the animals survived significantly longer in the ordinary well water.

7. When both the pH and the electrolytic content were practically equalized, there was no difference in survival time following irradiation.

The situation revealed by these experiments is not simple. Dismissing variation due to the physiological condition of the planarians, to differences in irradiation, and to temperature, at least four factors may be acting in the experiments summarized in Table 10 to affect the length of survival of the irradiated worms. These are: H-ion concentration per se, carbon dioxide concentration, total electrolytes present, and, perhaps, the ionic composition of the different waters used. The observed relations are schematically summarized in Table 11.

The first comparison in Table 11, *A*, taken alone, indicates that longer survival depends on a lower pH, more free CO_2 and more total electrolytes. The second comparison tends to rule out the importance of pH differences, at least in synthetic pond water; in fact, only in carbonate-free well water does pH seem to be a determining factor, and then longer survival was found in the more alkaline water (cf. Buchanan, 1930).

The first two comparisons in Table 11, *B*, indicate that the longer survival in synthetic pond water, as compared with well water, is a result of differences in concentration of total electrolytes. If these data represent the facts in the case, a relatively small difference in total electrolytes is effective. The other comparisons strengthen this conclusion concerning the importance of electrolytic concentration.

As will be shown in the next section, in waters having an electrolytic concentration such as is found in synthetic pond water, conductivity tests do not reveal any increase in total electrolytes when 100 planarians have lived in 100 cc. of calcium-free synthetic pond water for 4 hours. Since we are primarily concerned with the observed differential survival of grouped and isolated planarians following irradiation, these experiments raise immediately a question concerning the effect of groups on pH. With well water, which was the best buffered water we used, at total stage 30 we found that the water with about ten series of isolated worms had a pH of 7.8, while the accompanying groups were in water of pH 7.7 (difference, 0.1; $P = 0.003$). In nine other series at total stage 40 the same values were pH 7.7 and 7.3 (difference, 0.4; $P = 0.0001$). As far as they go, these differences would make for longer survival on the part of the grouped worms, primarily

because of an indicated increase in free carbon dioxide. There was little or no carbonate to be broken down in the synthetic pond water. Hence we should not expect to find a group-single differential so readily in this water; as shown in Table 5, this expectation was realized.

Effect of irradiation on permeability.—There is evidence that irradiation with ultra-violet increases permeability of living cells (Heilbrunn and Mazia, 1936). It is important in developing an explanation of the group protection from exposure to ultra-violet radiation to know the effect of the treatment on permeability of planarian worms. This was tested by measuring the change in total electrolytes as the worms remained in various

TABLE 11

A SCHEMATIC SIMPLIFIED SUMMARY OF THE DATA PRESENTED IN TABLE 10
(The Apparently Important Factor or Factors Are Italicized or Shown in Bold Face)

Water 1:2	pH 1:2	Free CO_2 1:2	Electrolytic Content 1:2	Ionic Constitution	Longer Survival
	A				
Well:Well	**7−:7+**	***more*:less**	***more*:less**	approx. same	1
SP:SP	**7−:7+**	***approx. same***	***approx. same***	***approx. same***	equal
CO_2-free:CO_2-free	**7−:7+**	approx. same	approx. same	approx. same	2
	B				
SP:Well	**7−:7+**	***less*:more**	***more*:less**	***different****	1
SP:Well	approx. same	less:***more***	less:***more***	***different***	2
CO_2-free:Well	**7−:7+**	***less*:*more***	***more*:less**	***different***	1
CO_2-free:Well	approx. same	***less*:more**	***more*:less**	***different***	1
CO_2-free:Well	approx. same	less:***more***	less:***more***	***different***	2
CO_2-free:Well	***approx. same***	less:more	***approx. same***	different	equal

* The difference between "SP" and "Well" is great, both quantitatively and qualitatively. Except for the absence of carbonates in one, the two types of well water differ only in quantity of certain ions.

waters after irradiation, as indicated by measurements of conductivity made by standard methods.

The usual calcium-free artificial water (p. 121) had a conductivity of 325×10^{-6} mhos, while a similar amount of the same water in 100 cc. of which 100 nonirradiated planarians had stood for 5 hours had a conductivity of 319×10^{-6}. In a parallel test with double-distilled water the blank tested 2.9×10^{-6}, and the water with worms showed 14.5×10^{-6}. Water collected from seven groups of 10 irradiated worms and from 20 single animals, prior to "30," showed no increase in electrolytic content over that found in the blank. Apparently neither irradiated nor normal worms give off electrolytes into artificial pond water; more comprehensive tests are given in Table 12. Two lots of 100 cc. solution—one with, and the other without, 100 normal worms—were left in 150 cc. Erlenmeyer flasks for 4 hours. This was a common length of time for irradiated worms to have begun cytolysis. At that time conductivity measurements were made. Again the results show that in full-strength calcium-free synthetic pond water there is

no gain in conductivity after planarians have lived in the water for 4 hours; rather there appears to be a small loss, as though something had been taken from the water. With increasing dilution there is a fairly steady increase in conductivity.

As a result of these tests, all further experiments on these points were made in double-distilled water.

Preliminary experiments indicated that the irradiated worms gave off electrolytes more rapidly than did nonirradiated ones. Accordingly, four experiments were made to follow the progressive loss of electrolytes into double-distilled water by lots of irradiated and untreated (control) worms. In Experiment C7, 500 worms were placed in 1,000 cc.; in C8, 200 worms were used per 300 cc.; in C9 and C10, 100 large worms were put in 100 cc. The worms in C9 were large (16–18 mm. long) and were sufficiently resistant so that they did not cytolyze according to the usual pattern following ultra-violet irradiation; the disintegration which finally took place was more like that found in the controls

TABLE 12

TESTS OF THE LOSS OF ELECTROLYTES FROM NORMAL WORMS INTO CALCIUM-FREE ARTIFICIAL POND WATER OF FULL STRENGTH AND OF VARIOUS DILUTIONS

Percentage of Pond Water	Blank 10^{-6} Mhos	Experimental 10^{-6} Mhos	Gain in Mhos
100	333.8	331.6	.....
57.3	204.9	201.4	.....
33.3	119.1	123.2	4.1
16.6	63.9	66.4	2.6
8.3	34.8	40.6	6.8
0.0	3.31	11.6	8.2

as a result of exposure to hypotonic water. In C10 the dosage was increased so that the irradiated worms showed the usual effects.

Except in Experiment C9, where it will be recalled that the dosage was insufficient to bring about cytolysis from ultra-violet irradiation, the loss of electrolytes is initially greatest in the irradiated worms. This increased rate of loss starts before there is any visible sign of an actual lesion. With the beginning of observable lesions, the rise in conductivity, that is, the loss of electrolytes, is greatly increased. Even in Experiment C9 the tendency toward greater loss by irradiated worms during the first few hours is noteworthy; and since it is a trend covering some five readings, it probably indicates an initial effect of the irradiation from which the worms later recovered.

Is calcium the protective factor?—Results summarized in an earlier section demonstrate that $CaCl_2$, even in extremely dilute solution (0.000001 M), delays the onset of cytolysis following ultra-violet irradiation. The work given in the last section shows that after such irradiation, tissues of planarian worms, on being placed in distilled water, are more permeable to contained electrolytes than are nonirradiated controls. The loss of electrolytes becomes much more rapid when actual lesions appear. These observations raise an obvious question: Is the accumulation of calcium around the grouped animals the factor which produces the observed group protection?

Unless otherwise noted, the results have been obtained by a titrimetric method which

is a combination of the reagents and procedures in several calcium oxalate methods of analysis (Peters and Van Slyke, 1932; Van Slyke and Sendroy, 1929; American Public Health Association, 1933).

Specifically, 500 cc. of the water to be analyzed was boiled down in a flask to about 75–100 cc. At some point during the boiling, 1 cc. of concentrated HCl was added to break down the carbonates. When the boiling was completed, the flame was lowered to maintain a simmer. Twelve drops of bromcresol green indicator (0.016 per cent solution) were added; then 2 cc. of ammonium oxalate; and, lastly, enough 1:1 NH_4OH to change the solution from yellow to a clear blue. This gave a pH of about 5 or more. At this pH calcium oxalate is precipitated. If indicated, more ammonium oxalate may be

TABLE 13

PROGRESSIVE LOSS OF ELECTROLYTES INTO DISTILLED WATER BY NORMAL AND BY IRRADIATED WORMS (TIME AT WHICH THE FIRST CASE OF CYTOLYSIS WAS OBSERVED MARKED BY ASTERISK)

(All Values in Mhos$\times 10^{-6}$)

Time in Hours	Expt. C7		Expt. C8		Expt. C9		Expt. C10	
	Normal	Irradiated	Normal	Irradiated	Normal	Irradiated	Normal	Irradiated
0.0.....	5.2	5.2	3.9	3.9	3.9	3.9	5.4	5.4
0.1–0.4.....	5.3	5.7	4.4	5.2	4.9	6.7	5.1	8.9
0.75–1.0.....	5.8	6.0	5.6	7.3*	.....	.....	5.3	7.5
1.3–1.5.....	6.5	7.0	6.1	8.2	7.1	7.5	6.2	8.7*
1.6–2.0.....	7.0	7.7	6.4	8.9	7.2	8.5	6.8	10.3
2.5–3.0.....	7.8	9.5*	.....	.....	8.8	8.9	8.3	14.5
3.8–4.5.....	9.7*	12.6	9.8*	18.1	10.3	10.8	.....	.....
5.0.....	10.6	14.7	.....	.....	.....	.....	12.2	25.1
6.0.....	12.5	17.3	.....	.....	13.8	13.7	13.5	30.6
6.8–7.0.....	14.2	20.1	13.9	24.1	15.3	13.7	.....	.....
8.0.....	16.9	21.7	.....	.....	15.7	14.3	.....	.....
18.8–23.2.....	36.3	36.0	41.8	39.5	44.1*	34.7*	38.8*	53.6
30.....	40.1	37.8	.....	.....	51.8	52.2	.....	.....
44.5–45.5.....	65.6	64.4	59.8	69.6	75.4	60.0	.....	.....

added. The mixture was then shaken occasionally and allowed to simmer for 10–15 minutes. When the precipitate settled readily after shaking, it was filtered through a No. 40 Whatman ashless filter. The filtrate was tested by addition of ammonium oxalate to assure the complete removal of calcium. About 50 cc. of a 2 per cent solution of NH_4OH was heated and used in four portions to wash down the precipitate in the filter paper. Complete washing-out of all excess ammonium oxalate is of great importance.

Fifty cubic centimeters of normal H_2SO_4 were heated to boiling and poured in portions over the filter paper containing the precipitated calcium oxalate while this was spread over the side of a beaker. The solution of oxalic acid so formed was transferred to a 125-cc. Erlenmeyer flask, heated to boiling, and titrated hot with N 0.05 (or N 0.01) potassium permanganate. Analysis of the amount of calcium in synthetic pond water showed that the titrimetric method outlined above gave 22.2, 21.2 (mean 21.7) mg. per liter. The gasometric method of Van Slyke and Sendroy gave 22.6, 19.9, 22.7, and 19.5 (mean 21.2) mg.; and a titrimetric modification of this method gave 23.3, 21.75,

and 21.50 (mean 22.3) mg. The calculated amount of calcium in this water was 21.9 mg. per liter. A test solution containing by weight 4.007 mg. of calcium per liter yielded 4.01, 4.11, 4.29, 5.12, 4.60, and 4.57 (mean 4.48) mg. per liter. One containing about 0.04 mg. of calcium per liter yielded 0.96, 0.84, and 0.65 (mean 0.82) mg. The previous test in distilled water had given 0.126 mg. per liter. Apparently the washing-off of the ammonium oxalate from the precipitated calcium oxalate had been incomplete. This error is hard to avoid.

Within a range of from about 22 to 4 mg. per liter this titration method is fairly reliable.

Tests of the amount of calcium given off by planarians into distilled water were then made. Five hundred worms irradiated 2 minutes at 26.6 cm. were placed in 500 cc. of double-distilled water. Eight hours later they averaged one lesion each (equivalent of total stage 30 of the usual assay tests); the distilled water blank showed 0.066 mg. for the 500-cc. sample; and the water decanted from the cytolyzing worms gave 0.50 mg. per 500 cc. Another lot of 250 worms were irradiated and placed in 250 cc. of double-distilled water. They cytolyzed to mean stage 3 in 10 hours. The accompanying distilled water blank showed 0.056 mg. of calcium, and water decanted from the worms contained 0.225 mg. per 250 cc.

From these data it appears that the amount of calcium lost into distilled water by 500 cytolyzing worms in 500 cc. of water is on the order of 1 mg. per 1,000 worms per liter, a value which lies below the tested limit of the method used. Considering the determination as valid, this would amount to about 0.001 of a milligram per worm.

The method used is relatively reliable at a level of 4 mg. calcium per liter. It is perhaps noteworthy that the solution from the analysis of worm water showed a faint cloudiness after the intended precipitation of the calcium as oxalate, a condition which is lacking in the various blanks. Hence, we may not have gotten quite all the calcium that was present.

It is evident that we were unable to find or develop methods of analysis which would allow us directly to test the hypothesis that the observed group protection results from the greater addition of calcium by the group. If we accept the results obtained at their face value, by calculation we would expect that for the group at total stage 30 there would be approximately 0.01 mg. of calcium in 5 cc. of water, while the water around the associated isolated planarians would contain only 0.001 mg.

From direct experimentation we know that 0.000001 M $CaCl_2$ does retard the onset and progress of cytolysis following irradiation. This amounts to an increase of 0.04 mg. of calcium per liter, or to 0.0002 per 5 cc.; hence it would be expected that the calculated increase of 0.01 mg. in 5 cc. of distilled water could result in a decreased rate of cytolysis as compared with water containing one-tenth that amount.

Any general theory that the observed group protection in well water and alkaline synthetic pond water is a result of calcium leached from the grouped worms runs against the observation that there is no measurable change in the electrolytic content of such water after worm cytolysis. There are other objections to considering protection as a result of increased calcium content to be the entire explanation of the observed phenomena. These have already been presented and will be reviewed later. Now it is necessary to consider another differential factor.

The effect of carbon dioxide on the rate of cytolysis.—In order to test the effect of increased carbon dioxide on the onset and progress of cytolysis of irradiated planarians, a

number of experiments were set up as follows: Six series of eleven saltcellars were arranged, each containing the usual 5 cc. of water. Well water was used throughout. A twelfth dish was added which contained the same amount of water plus Hellige indicator. Each series was placed in a covered crystallization dish, three of which were charged with properly washed carbon dioxide from a Kipp generator until the indicator dish showed a pH of 6.0. The worms were irradiated as usual in lots of 20 per Petri dish. Two such lots, as nearly equal in size and other relations as possible, were distributed one half of each lot to an experimental and the other half to a control series. The six series gave three paired comparisons for each experiment.

The three experimental chambers were recharged with the gas after the animals were set out, and then again at or near total stage 30, for the obvious reason that carbon dioxide readily escapes if exposed to ordinary atmosphere. Appropriate controls showed that nonirradiated worms were not visibly affected by such carbonated water. The results from a number of paired series, each of which contained in all 500 worms are summarized in Table 14.

TABLE 14

THE EFFECT OF CARBON DIOXIDE ON THE RATE OF CYTOLYSIS
(Each Comparison Based on 25 Paired Series)

Comparison*	Minutes to "30"	Difference	*P*	Percentage	Difference	*P*
WGG:WGS.....	169:123	46	0.038	100:86	14	0.0012
CDGG:CDGS.....	225:195	30	0.017	100:87	13	0.0014
CDGG:WGG.....	225:169	56	0.025	100:79	21	0.0018
CDGS:WGS.....	195:123	72	0.003	100:77	23	0.0002
CD:W.....	210:146	64	0.006	100:78	22	0.0002

* "W," well water control; "CD," carbon dioxide treated water.

The group-single differential appears significantly in both the well water controls and the carbon dioxide experimental series. The worms treated with carbon dioxide, both grouped and isolated, survived significantly longer than did similar ones placed in well water.

DISCUSSION

In general terms our knowledge concerning the effect of numbers on different biological processes can be summarized in the two curves shown in Figure 1, in which distance above the base line indicates the speed of some biological process, or group of processes, and distance to the right shows increasing population density.

Some aspects of mass relations show no effect of undercrowding (Fig. 1, *A*); such biological processes are typically retarded with increasing numbers. The experience of Pearl and Parker (1922) with effects of numbers of pairs of *Drosophila* on the rate of egg-laying per female is a well-known case in point. Other phases of group relations are summarized in Figure 1, *B* (cf. Allee and Bowen, 1932). In these there is a maximum increase in some physiological relationship at some distance above the minimal numbers possible, usually well before the maximum population is reached.

As Allee (1938) says, it would be better perhaps, in so far as this is possible, to view population effects in terms of these descriptive curves rather than as harmful or bene-

ficial results. Such terms, if used to interpret survival values only, are relatively objective; they are, however, somewhat ambiguous and can be interpreted in a more anthropomorphic manner.

The effects of undercrowding, shown by the left-hand limb of the curve in Figure 1, *B*, have been reported for a wide variety of organisms and conditions. Many of these cases have been reviewed elsewhere (Allee, 1931, 1934, 1938), and no attempt will be made to list or discuss other pertinent cases which are to be found in the widely scattered and growing literature on the subject. It suffices our present purpose to say that in the present state of knowledge a first concern in undertaking a study in population physiology is to find whether the phenomena under investigation belong to those which can be summarized by curves related to Figure 1, *A*, or to Figure 1, *B*. After settling this point, a second and more difficult problem is concerned with the analysis of the factors involved in producing the observed results.

The case reported in the early sections of this paper, that of group protection for *E. dorotocephala* during and after exposure to ultra-violet radiation, belongs obviously

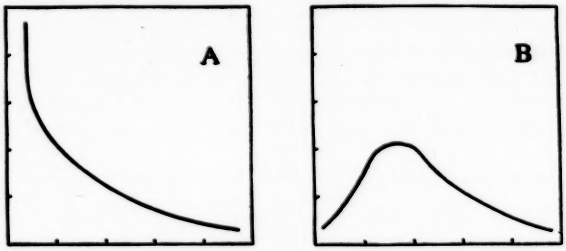

FIG. 1.–Curves summarizing the effects of numbers present on biological processes

to the *B*-type of curve. At the dosages used, few of the planarians survived indefinitely. They were purposely irradiated sufficiently so that such would be the case. With a shorter exposure, which was sometimes unintentionally given, we could have found a dosage at which all the isolated worms would be killed off while many, sometimes the majority, of the grouped worms would recover. Having established the existence of undercrowding under these conditions, we then proceeded to the harder task of trying to find the mechanism or mechanisms involved.

It is pertinent in this connection to note again that immediately after irradiation the planarians were transferred to fresh, that is to say, nonirradiated, water to await cytolysis. This transfer after irradiation was made by changing one or a few worms to the group and then isolating a similar number.

We know that being left in the water in which they were irradiated hastens the beginning of cytolysis. In test series half were transferred immediately as usual, and half after a delay of 5 minutes. Both grouped and isolated worms cytolyzed more rapidly if the transfer was delayed. In the given instances they survived (to total stage 30) 26 per cent shorter time with $P = 0.042$.

In the usual group-single tests there was more irradiated water introduced into the water about the group than into that about any given isolated worm; hence, the observed group effect was not a result of the grouped worms being exposed to less of the irradiated

water. Whatever may be the cause of the longer survival of the grouped planarians, it is evidently not the result of a differential response produced by the amount of irradiated water which was accidentally carried over.

Heilbrunn and Mazia (1936) summarized the somewhat scanty evidence which indicates that treatment with ultra-violet increases permeability. When tissues of many layers are so treated, they recognize that cytolytic changes, rather than reversible membrane effects, may be responsible for the observed changes. The data we have presented, which show that irradiated planarians lose electrolytes into distilled water more rapidly than do nonirradiated controls, do not throw light on this particular point. The electrolytes can be demonstrated to have leached out before lesions were visible on the living worms.

The effects on water produced by worms living or dying and disintegrating give what we call "conditioned water." Tests of the effect of such water on the rate of cytolysis show that, at least as tested, the effect of heavily conditioned water hastened cytolysis, as did more lightly conditioned water if it produced any measurable effect. This is evidence against the observed retardation of cytolysis among grouped worms being a result of the action of something added to the water by the disintegrating worms. Calcium so introduced into worm-conditioned fresh water was shown by Oesting and Allee (1935) to account for the greater longevity of the marine *Procerodes* isolated into hypotonic water. Other evidence at hand concerning the relations of calcium to disintegration of these planarians after treatment with ultra-violet rays can be now summarized and evaluated.

Calcium is known to decrease permeability of cell membranes; hence, if the primary effect of ultra-violet treatment is to increase permeability either by a reversible action on cell membranes or by destructive irreversible processes, a substance which acted to preserve or decrease permeability would probably defer and retard cytolysis.

Irradiated planarians in distilled water disintegrate more rapidly than in the other waters used, all of which contain relatively large proportions of calcium, or of magnesium, which also retards cytolysis in these worms. Cytolysis took place more slowly in well water with 40–50 mg. of calcium per liter than in synthetic pond water of the same pH. The calcium content of such water was approximately half that of well water. The irradiated planarians also survived longer in synthetic pond water containing calcium than in similar water in which magnesium had been substituted and which contained no calcium. When $CaCl_2$ was placed in well water to make a 0.0083 M solution (disregarding the calcium already present), the planarians survived decidedly longer than in plain well water. This amounts to adding some 334 mg. of calcium to the amount already present and is out of all proportion to the amount added by 10 disintegrating worms in 5 cc. of water.

With the worms in distilled water the protective value of 0.000001 M $CaCl_2$ could be demonstrated. In this water there was an increase of 0.04 mg. calcium per liter. While we did not find or develop quantitative methods which would accurately determine the amount of calcium present at this dilution, we did get indications that 500 planarians in 500 cc. of water gave off on the order of 0.5 mg. of calcium. If the yield was at the same rate when 10 worms were placed in 5 cc., we could expect that there would be 0.01 mg. of calcium present, which should retard cytolysis, as compared with that shown in water

containing an isolated worm. For distilled water, then, calcium may be one of the factors that caused the observed group-single differential survival.

With well water or the synthetic pond water used, this can hardly be an important agent, since, as was stated earlier, in such waters the conductivity was not measurably changed by the presence of disintegrating worms, originally 1 worm per cubic centimeter. To be sure, a decided increase in the amount of calcium in well water increased survival of the planarians; however, the amount of calcium introduced was out of all proportion to the amount given off by worms that cytolyzed in distilled water.

The observations recorded earlier show that worms disintegrate more slowly in well water with pH below 7.0 than in more alkaline well water. This water is rich in carbonates. For years we have known that an increase in acidity in water with carbonates may produce effects either directly as a result of the H-ion concentration or as a result of the release of carbon dioxide (Smith and Clowes, 1924). From data given in Table 10 we find that only in carbonate-free well water is pH the apparently effective factor, and here the survival is longer in the lower H-ion concentration. This factor had no effect on survival in synthetic pond water which lacks carbonates. The longer survival in carbonate-containing well water of higher H-ion concentration is probably related to its action in setting free carbon dioxide.

In comparing different types of water of the same or different pH, it becomes clear that total electrolytic concentration, when artifically varied, is an important factor. Length of survival is directly related to the conductivity of the water. It has been demonstrated that animals increase the total electrolytic content when placed in distilled water. In such water, therefore, this could be a mechanism resulting in group protection. Loss of electrolytes into waters of high conductivity has not been shown.

Direct experiments show that well water charged with washed carbon dioxide to pH 6.0 caused worms placed in it to survive the effects of irradiation longer than in well water controls at pH 7.3 or higher. Both these initial pH values drifted. After 3.5 hours, when the worms were approaching total stage 30, the carbon dioxide charged well water was about pH 7.8, while the straight well water showed pH 8.2.

As a result of these observations we have three different mechanisms which may have operated in at least some of the cases in producing the observed differential survival of grouped and isolated animals. In distilled water the larger amount of calcium and other electrolytes leached from the grouped planarians may have been effective in retarding cytolysis. In distilled water and in all other waters used, the more rapid accumulation of carbon dioxide would have the same effect, while in some waters, the higher H-ion concentration about the group may have acted indirectly to lengthen the survival of grouped worms. Much more work would be needed to establish the exact limitations of the role of each of these factors in producing the observed group effect.

The case reported in the earlier sections of this paper, that of group protection for *E. dorotocephala* during and after exposure to ultra-violet radiation, adds another to the list of recently demonstrated examples of undercrowding, as summarized by the left-hand limb of the curve in Figure 1, *B*. This is not the place to discuss the general biological implications of these particular experiments or the phenomena associated with undercrowding as a whole. Certain aspects of this discussion have been undertaken recently by the senior author (Allee, 1938), and we have neither space nor inclination to repeat or summarize this material.

SUMMARY

1. *Euplanaria dorotocephala* can survive exposure to ultra-violet radiation longer, other things being equal, if exposed in some numbers, even though no shading occurs, than if isolated while being irradiated. The worms were always assayed after transfer to fresh water which had not been irradiated.

2. After identical irradiation, groups of 10 planarians survive longer after transfer to a small amount of fresh water than do similar planarians isolated into the same amount of water.

3. As might be expected, planarians irradiated in lots of 10 and transferred together to fresh water survive much better than if kept continuously isolated during and following irradiation.

4. Worm-"conditioned" water, as tested, hastened the cytolysis of irradiated worms.

5. The addition of $CaCl_2$ delayed cytolysis; in double-distilled water a solution as dilute as 0.000001M was appreciably effective.

6. The group effect was apparent when the grouped and isolated worms were placed in proportional volumes in the usual saltcellar embryological dishes. It disappeared when volumes were proportional and the depth was equal.

7. Agitation of the worms hastens cytolysis and causes the differential survival of grouped and isolated worms to disappear.

8. Planarians tend to cytolyze more slowly after irradiation if placed in well water with pH between 6.0 and 6.8 than if in water with pH of 7.3–8.2. This difference is not found in synthetic pond water. The reverse occurs in carbonate-free well water.

9. Irradiation increases the permeability of planaria to contained electrolytes if the worms are placed in hypotonic water; no effects were found in waters with a conductivity of 325×10^{-6} mhos or more.

10. Grouped planarians probably give off enough calcium into distilled water to retard cytolysis significantly; this is probably not a factor with waters already fairly rich in calcium.

11. An increase in carbon dioxide also delays cytolysis after irradiation.

12. We are not prepared to delimit precisely the roles played by calcium, total electrolytes, H-ion concentration, and carbon dioxide in producing the longer survival of grouped planarians.

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REACTIONS OF TADPOLES TO LIGHT. I. EFFECT OF CHEMICALS ON AGGREGATION OF TADPOLES IN UNILATERAL ILLUMINATION

(Eight figures)

H. T. FOLGER
Biological Laboratory, Fisk University

AN ATTEMPT to find out if tadpoles vary in reactivity to light led to the experiments to be described in this paper. In order to carry out this work, it was necessary to obtain a series of measurements that would express quantitatively the degree of sensitivity to illumination and thus permit an exact comparison of their reactivity at different ages. It soon became apparent, however, that there is little or no relation between age and sensitivity. Tadpoles that have just left the mass of jelly that surrounds the eggs are, perhaps, generally less positive than older ones; but, aside from these very young individuals, no evidence of a correlation between age and sensitivity to light was observed.

One thing, however, was evident from the very beginning of the experiments: the sensitivity of tadpoles may differ markedly from time to time, even if not in a progressive manner. A group that is strongly positive today may be much less so or, rarely, may be even negative tomorrow, a behavior which suggests that in their response to illumination these animals are easily affected by the environment. Consequently, they should be excellent subjects for a study of the effects of various reagents on the response to light. It was for this purpose that they were used in the experiments herein recorded.

It has long been known that numerous factors may exert an influence on the response of organisms to light. Of these, perhaps the most important is the intensity of the light itself. Many organisms are positive at one intensity and indifferent or negative at another. Temperature has a marked effect on the reactions of most, and perhaps all, organisms; and, among those that live in the water, the chemical condition of the medium is usually of the utmost importance. But, although these things may have so pronounced an influence, there is a remarkable lack of specificity of effect. One can scarcely make a single generalization that will apply in all cases. Increase in temperature may cause negative animals to become positive; but, on the other hand, it may cause positive animals to become negative. Acids make negative *Spondylomorum* positive, and positive ones still more positive (Mast, 1918); but, as we shall see, they make positive tadpoles negative, and negative ones more negative. Numerous examples of such contrasting results could be cited. In spite of the fact that many data have already been collected, it is undoubtedly true that observations on various additional organisms will have to be made before definite and final conclusions can be drawn as to the effect of almost any reagent. It was in the hope of adding something toward the elucidation of the effects of a few reagents that the investigation to be recorded was undertaken. It deals with the action of certain chemicals on the response to light by *Bufo* and *Rana* tadpoles.

METHODS

If one is to relate the reactions of an organism at different times and under diverse conditions, it is obviously advantageous to use some system of quantitative measurement that will admit of accurate comparison. Kanda (1919), in his work on *Arenicola*

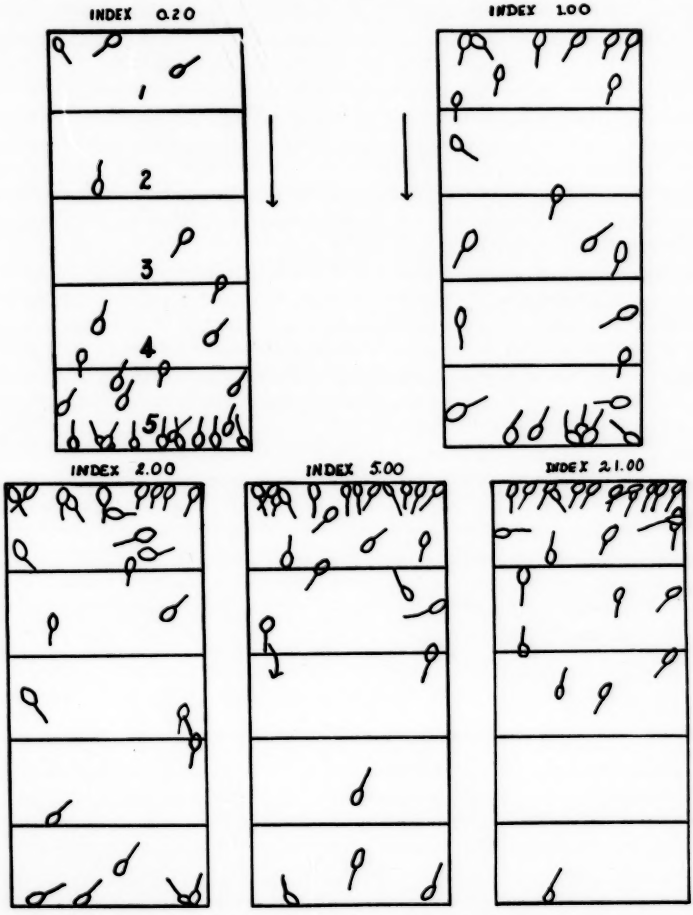

FIG. 1.—Illustrating distribution of tadpoles in dish at various indices of reaction. Direction of light indicated by arrows. (See text.)

larvae, recorded estimates of the percentages of larvae that were attracted to the lighted end of a dish; and these estimates are of more aid to the reader than such expressions as "strongly positive" or "slightly negative." A somewhat more exact method of measurement has been employed in the present instance and will be described below.

Groups of 25 tadpoles were put into 1,000 cc. of water in rectangular museum jars, 12 inches long by $3\frac{1}{2}$ inches wide by 8 inches high, and subjected to illumination, which was furnished by a 200-watt concentrated filament lamp, situated about 8 inches from one end of the jar. Under the latter was placed a piece of black cardboard, $12 \times 3\frac{1}{2}$ inches in size, which was divided into five equal parts by means of white cross-lines. When viewed from above, the white lines stood out conspicuously on the black background and served to divide the jar into compartments. These compartments were numbered from 1 to 5, 1 being nearest the light and 5 farthest from it. The type of reaction could thus be ascertained by comparing the numbers of tadpoles in the various compartments. In some instances, especially when results are expressed in curves, it has been convenient merely to indicate the number of tadpoles at the lighted end of the jar, that is, in compartments 1 and 2. This, however, does not admit a ready comparison of groups of different numbers; and to facilitate such comparison and to condense and simplify results for presentation in tables the following scheme was devised. The number of tadpoles in compartments 1 and 2 were added (*a* of Table 1), and the sum divided by the number in compartments 4 and 5 (*b* of Table 1).[1] The resulting quotient has been called the "index of reaction." It is evident that an index of 1 shows a group as a whole to be indifferent to light, there being then as many animals at one end as at the other. An index of more than 1 indicates a positive group; and of less than 1, a negative group.

In order to help the reader to visualize the relations indicated by the index, a series of diagrams is presented in Figure 1, in which the number of tadpoles in the several compartments is shown, with indices of 0.20, 1.00, 2.00, 5.00, and 21.00, respectively.

Three different species of tadpoles were used in the experiments: toad (*Bufo*), wood frog (*Rana cantabrigensis*), and leopard frog (*Rana pipiens*). There appears to be no specific differences in their behavior, as far as reaction to light is concerned. Anything that affects the responses of one species apparently has the same general effect on the responses of the others. However, because their excessive activity somewhat hindered accurate counting, the tadpoles of the toad were not quite so favorable for study as the others. Those of *R. pipiens* were perhaps the best, but all three of the species could be used and were used to advantage.

EXPERIMENTAL RESULTS

A. PRELIMINARY EXPERIMENTS

Before considering the effects of chemicals on the reaction to light, it may be of some benefit to inquire briefly into the general behavior of tadpoles when they are placed in tap water in a rectangular jar (previously described) which is illuminated from one end.

1. *Variation.*—As one would expect, there is some variation in the reactions of a given group over a period of time, and there may be a very considerable diversity in the reactions of different groups. Examples of such variations are shown in Figure 2, in which are recorded observations on two lots of 25 individuals each. The observations, which were taken every minute, extended over a period of more than half an hour. The

[1] Note that the tadpoles in the middle compartment are ignored, as they indicate neither a positive nor a negative condition. It would have simplified results somewhat if the dish had been merely divided into two parts. The larger number was chosen at the beginning of the experiment, with the prospect that a more detailed record might be of some importance. It was retained because, since a very considerable amount of data was collected with it, it would offer a better comparison of data collected later.

curves shown in the figure denote the numbers of animals at the lighted end of the jar, in compartments 1 and 2. Obviously, there was a difference in the sensitivity of the two groups. One was decidedly positive, as indicated by an average of 20.8 tadpoles at the lighted end. The smallest number at this end at any time during the entire experiment was 16, and the largest number was 24. The second group, on the other hand, was practically indifferent, with an average of 11.6, a minimum of 8, and a maximum of 13 at the lighted end. While the two groups differed every considerably from each other, there was not a very marked deviation in the reactions of either. The average number from almost any five consecutive observations would be very close to the average from

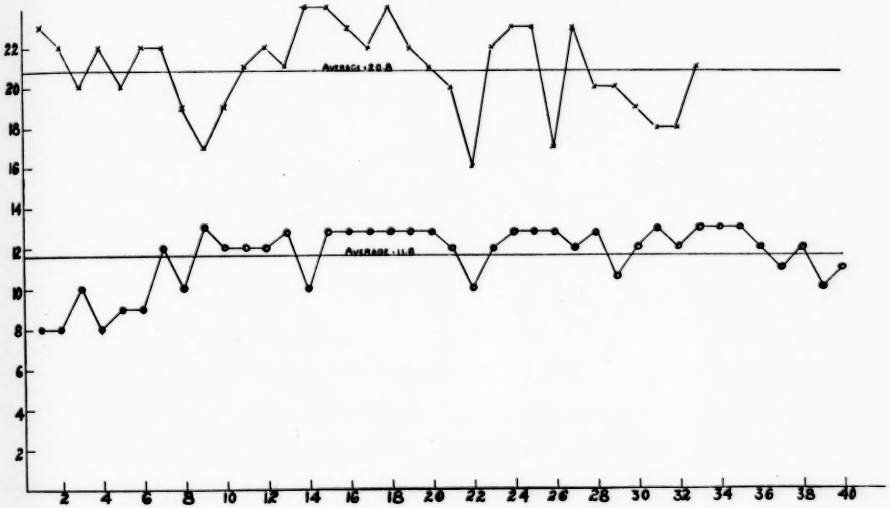

FIG. 2.—Showing variation in numbers at lighted end of dish in two groups of tadpoles over a considerable period of time. Abscissas, time in minutes; ordinates, number of tadpoles (out of a possible 25) at lighted end of dish

all the observations. In experiments to be described, averages have been established from five observations.

As far as variation within individual groups is concerned, the foregoing examples are typical. In very few instances was it found to be materially greater or less. Neither lot, however, could be said to be typical with respect to the quality of reaction, that is, to the degree of positiveness or lack of positiveness with which it responded to light. As a matter of fact, the divergence in the reactions between different groups was so marked that any one group could scarcely be designated as typical. In tap water a few of the many groups experimented with were slightly negative. In general they were positive.

2. *Time required for aggregation.*—From the foregoing results it is evident that tadpoles distribute themselves in a certain way over a dish that is illuminated from one end, and, if undisturbed, tend to retain this distribution. An alteration in the environment, such as a change in the chemical content of the surrounding medium or in the direction

of the light rays may lead to a redistribution. In Figure 3 are shown the results of an experiment designed to ascertain the time required for reaggregation after the direction of the light was reversed. In this experiment two 200-watt lamps were placed at opposite ends of a jar. Alternately one was turned on for a few minutes and the other turned off. Twenty-five tadpoles were put in the jar, and observations were made every minute. The numbers recorded are those of animals in the two compartments at the left end of the dish. As indicated in the figure, the left light was first turned on, and three observations made. The first observation showed 17 animals at the left end; the second, 19; and

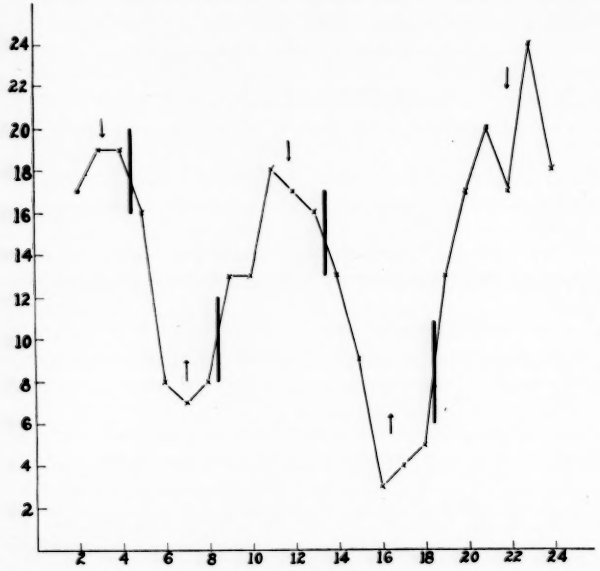

FIG. 3.—Showing time for reaggregation when direction of light is reversed. Lamps placed at both ends of dish, and first one turned on, then the other. Heavy vertical lines indicate time at which light was changed. Abscissas, time in minutes; ordinates, number of tadpoles at left end of dish. Arrows indicate direction of light.

the third, 19. The left light was then turned off, and at the same time the right one was turned on. Four observations showed 16, 8, 7, and 8 animals, respectively, at the left end. Again the lights were reversed; and five observations found 13, 13, 18, 17, and 16 animals at the left end, which was now again the illuminated end. As the figure indicates, two more reversals of the light gave the same results, namely, a redistribution of the tadpoles within 1 or 2 minutes after the position of the light was changed.

B. EFFECT OF CHEMICALS

1. *Eosin.*—Of the various reagents whose reactions were investigated, eosin proved to be one of the most efficient in influencing the reaction to light. Its effects are clearly shown in Table 1. Since the results set forth in this table also illustrate the general

method of procedure in several subsequent experiments, it will be well to examine them somewhat in detail. As indicated, six different lots of tadpoles were used, one of them twice. In the case of each lot, five, and in one or two instances four, observations were made at intervals of about 15 minutes, and the number of animals in the several compartments was ascertained. Enough eosin was then added to give a decided tinge to the water; and, after 30 minutes, five more readings were made, with the same time-intervals as before. The jar was reversed after each reading. The figures in the table are the averages of these various readings.

Group 25 might be selected as an example fairly illustrating the effect of eosin. Before treatment there was in this group an average of 15.5 tadpoles in compartments 1 and 2,

TABLE 1

SHOWING THE EFFECT OF EOSIN ON THE REACTION TO LIGHT BY TADPOLES

Each group was composed of 25 animals (21 in the case of No. 101). Treatment consisted in adding sufficient eosin to give a marked color to the water containing the tadpoles. Details of procedure are given in the section "Methods."

No. of Group	Nos. of Animals in Various Compartments										Index of Reaction (a/b)	
	Before Treatment					After Treatment						
	a			b		a			b			
	1	2	3	4	5	1	2	3	4	5	Before Treatment	After Treatment
101 (wood frog)	9.8	2.6	2.0	2.4	4.2	0.8	0.8	1.2	3.0	15.2	1.88	0.09
25 (wood frog)	14.25	1.25	2.5	2.0	5.0	3.75	0.25	0.0	1.25	19.75	2.21	0.19
111 (wood frog)	14.8	1.8	1.2	1.6	5.6	1.2	0.6	2.0	2.6	18.6	2.31	0.08
111 (wood frog)	6.8	2.6	2.6	4.8	8.2	0.25	0.25	0.75	5.75	18.0	0.72	0.02
104 (toad)	16.8	1.8	2.6	1.6	2.2	10.4	2.6	3.2	1.6	7.2	4.89	1.48
109 (toad)	10.0	3.4	1.6	1.6	8.4	6.4	2.8	1.8	3.6	10.4	1.34	0.66
107 (*Rana pipiens*)	6.2	2.8	2.8	3.4	9.8	1.2	0.8	3.4	5.2	14.4	0.68	0.10
Average	11.2	2.3	2.2	2.5	6.2	3.4	1.2	1.8	3.3	14.8	1.55	0.25

and an average of 7 in compartments 4 and 5, giving an index of 2.21. Introduction of the eosin brought about a conspicuous change in reaction. In compartments 1 and 2 the average number of tadpoles fell to 4, and in compartments 4 and 5, rose to 21, reducing the index of reaction to 0.19. Thus the dye had the effect of causing a decidedly positive group to become strongly negative. Examination of Table 1 will show a similar effect in every instance. Group No. 104 was very positive, with an index of 4.88. To be sure, eosin did not cause it to become negative, but it did greatly lower the positive reaction, decreasing the index to 1.48. Two groups, Nos. 111 and 107, were negative before treatment. In each instance eosin caused a further decrease in the negative reaction, reducing No. 111 from 0.72 to 0.02 and No. 107 from 0.68 to 0.10. All the other groups were changed from positive to negative. The average of all the groups before treatment was 1.55; after treatment, 0.25. Clearly, eosin tends to make positive tadpoles negative, and negative ones still more negative.

It has already been stated that untreated groups of tadpoles, that is, groups which have been in tap water that is slightly alkaline, are generally positive. Of the six groups we have just noted, four were decidedly positive before treatment, one was negative, and the other, No. 111, the only one that was used twice, was first positive and then negative. As indicated, No. 111 was first distinctly positive. Treatment with eosin made it negative, as one would expect. Left in the eosin overnight, it was removed to tap water 2 hours before its reaction to light was again tested; but 2 hours was not long enough for complete recovery from the effects of the dye. Given sufficient time in the tap water, it undoubtedly would have become positive again. Thus, only one group, No. 107, was

TABLE 2

SHOWING THE EFFECTS OF VARIOUS CHEMICALS ON THE REACTION TO LIGHT BY TADPOLES

Each experiment included ten observations, five before and five after the introduction of the chemical. For method of obtaining index of reaction, see Table 1.

Chemical Used	Number of Experiments	Index of Reaction		$\frac{x}{y}$
		Control (x)	Treated (y)	
Ammonium hydroxide	7	1.10	4.10	0.27
Sodium hydroxide	12	2.01	2.21	0.91
Urea	10	2.18	2.02	1.08
Ether	9	4.17	3.31	1.26
Sucrose	14	2.74	2.11	1.30
Sodium chloride	4	2.01	1.31	1.53
Dextrose	13	2.50	1.62	1.54
Ethyl alcohol ($\frac{3}{4}$ of 1 per cent)	14	2.48	1.43	1.73
Sulphuric acid	14	3.69	1.65	2.24
Methyl alcohol (1 per cent)	22	2.55	1.11	2.30
N-propyl alcohol ($\frac{3}{4}$ of 1 per cent)	9	3.64	1.45	2.51
Eosin	7	1.55	0.25	6.20

really negative before treatment. Experience has shown that the proportion of negative untreated groups is even much lower than indicated by these numbers.

It so happens that group No. 107 was composed of tadpoles of *R. pipiens*, the only tadpoles of this species used in the experiment. That it was negative was apparently merely fortuitous, as further observations have given no reason for believing that the tadpoles of this species are more likely to be negative than those of *R. cantabrigensis* or *Bufo*.

2. *Comparison of various chemicals.*—That eosin is not the only substance that appreciably affects the reaction of tadpoles to light is clearly apparent from Table 2, which shows the effects of the several chemicals that were investigated. The procedure in these tests was precisely like that in the experiment just described; that is, the reaction of the untreated group was first found, the chemical then added, and after half an hour the reaction again ascertained. As may be seen, ammonium hydroxide was the only substance used which caused a notable increase in the positive reaction. Seven lots of tadpoles were employed to ascertain the effect of this reagent. Before being subjected to ammonium hydroxide, several of them were made negative with eosin or acid; and as a consequence

the average index of reaction of the seven groups was low. The action of the ammonium was very pronounced. Every positive group was made much more positive, and every negative group was made positive. The general average was raised from 1.10 to 4.10.

Sodium hydroxide does not appear to be nearly so potent as ammonia in influencing the response to light. From the data presented in Table 2 there might even be some question as to whether it has any effect at all. The average reaction of the ten groups tested was 2.01 before treatment and 2.21 after treatment, a difference which is perhaps within the limits of experimental error. As a matter of fact, these figures are misleading. It will be demonstrated later that sodium hydroxide actually does have a marked influence on the reaction to light.

With the possible exception of urea, all the other chemicals that were tested tend to make positive groups less positive or negative, though with varying degrees of efficiency. And even urea lowered the index of the reaction from 2.18 to 2.02, a difference which is not great enough, however, to be of much significance. Ether lowered the index of the reaction from 4.17 to 3.31; sucrose from 2.74 to 2.11; sodium chloride from 2.01 to 1.31; and dextrose from 2.50 to 1.62. Various concentrations of these chemicals were employed, and the foregoing figures might be materially changed by picking out and using the concentration that is most effective. Three alcohols—methyl, ethyl, and propyl—were used. All three proved to be fairly efficient in reducing the index of the reaction, 1 per cent methyl changing it from 2.55 to 1.11, three-fourths of 1 per cent ethyl from 2.48 to 1.43; and three-eighths of 1 per cent propyl from 3.64 to 1.45. Sulphuric acid also lowered the index of the reaction rather markedly, namely, from 3.69 to 1.65. We have already noted the effect of the eosin.

To facilitate somewhat a comparison of the relative effects of the different substances that were used, the indices of the reaction obtained before treatment has been divided by the indices obtained after treatment, and the quotients placed in the final column of Table 2. It should be kept clearly in mind, however, that this comparison means little except in a very general way. Undoubtedly eosin, generally speaking, is more effective than sodium chloride or dextrose in causing tadpoles to become less positive to light. But, while 1 per cent methyl alcohol is evidently more efficient than three-fourths of 1 per cent ethyl, methyl can hardly be ranked above ethyl for that reason, as other concentrations might very likely have reversed their relative effectiveness.

3. *Sulphuric acid.*—Although in a large number of instances several of the foregoing chemicals, especially those at the bottom of the list, such as the alochols or sulphuric acid, made positive groups negative, it is evident from Table 2 that, with the exception of eosin, they generally did not: they usually made slightly positive groups negative, and strongly positive groups less positive but not negative. Eosin thus appears to be more potent in effecting a change in reaction than any other substance that was used.

"Appears" has been used advisedly in the preceding sentence, for further experience has shown that the most noteworthy effects of sulphuric acid, and ammonium and sodium hydroxides as well, are to be observed within a few minutes after the chemical has been introduced, and so cannot be discovered with a procedure such as the one described, in which no observations were made until 30 minutes after the chemical was added. This early effect of sulphuric acid is clearly illustrated by the results of an experiment which are presented in Figure 4. In this experiment 69 observations were made at intervals of 1 minute—23 of them before the acid was added, and the remainder after. A group of 25 tadpoles was used as usual. Time is represented in the figures by the

abscissas; and the number of animals at the lighted end of the jar, in compartments 1 and 2, by the ordinates. As may be seen, the group was decidedly positive to light, there being from 17 to 19 animals at the lighted end of the vessel. It remained strongly positive for 3 minutes after the introduction of the acid, then on the fourth minute the number at the lighted end suddenly fell to 4. The group had thus become strongly negative, and it continued in this condition for 6 minutes. Then it gradually became less negative, until on the seventeenth minute after the addition of the acid the number in the first two compartments had reached 14. The group was positive again, but only

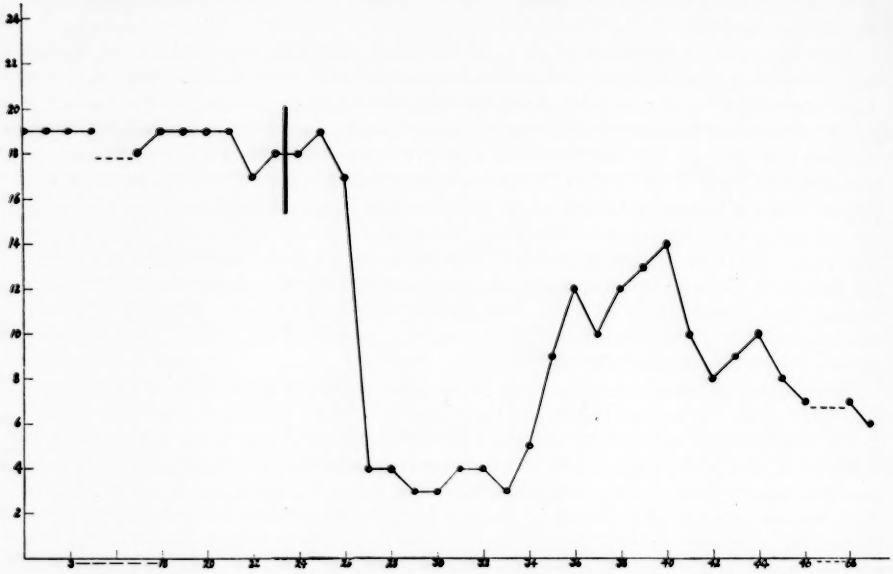

FIG. 4.—Effect of sulphuric acid on reaction to light. Abscissas, time in minutes; ordinates, number of tadpoles at lighted end of dish. Heavy vertical line indicates time at which acid was added to water containing the tadpoles.

temporarily, for the number of tadpoles at the lighted end began to decrease, until in a few minutes there were but 7 left; and this negative condition persisted for the remainder of the experiment.

The effect of acid on the reaction to light thus appears to be clear-cut. To show that the peculiarities just described are characteristic and not merely accidental, the reactions of four additional groups are indicated in Figure 5. All the curves in this figure were constructed as was the curve in Figure 4, and all denote the same type of behavior as described above. In addition, still other experiments were made, and almost invariably with the same general result. Within 1–4 minutes after introduction of acid there was a rapid lessening in the number of animals at the lighted end of the jar, sometimes, as shown in curves 2 and 3 of Figure 5, there being none at all left at this end. Then followed the curious postreaction, and the tadpoles surged back to the lighted end until

there were almost as many, or, in a few instances, fully as many, as before the addition of the acid. However, in a very short time (usually within 1 or 2 minutes) they began to retreat, with the final result, as noted above, that the group became either negative or much less positive than it was before treatment.

4. *Ammonium and sodium hydroxides.*—It has already been noted that ammonium hydroxide brings about a decided increase in the positive reaction. Ammonium hydroxide is like the acid, however, in that its most pronounced effects are to be observed shortly after its introduction into the water. This may be clearly seen from the experiments on two groups of animals, the results of which are presented in Figure 6. The procedure in these experiments was similar to that in the experiments with sulphuric acid just

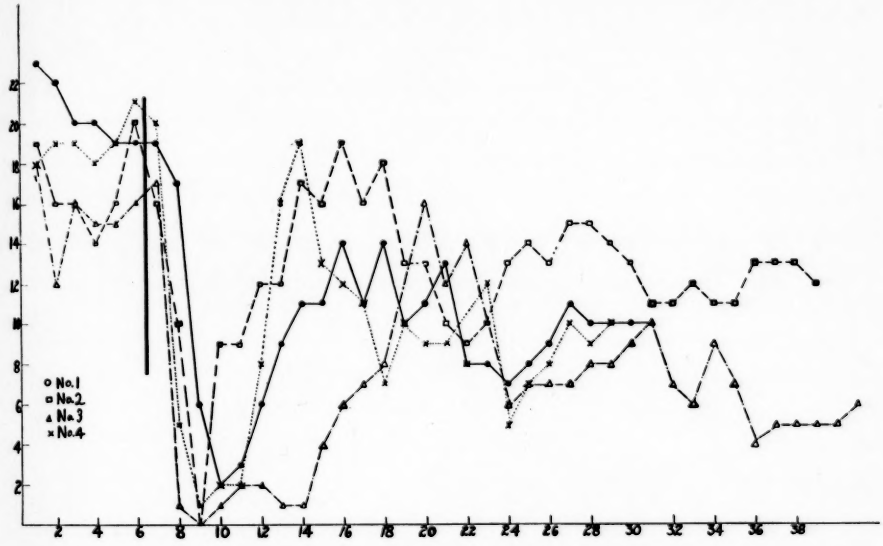

FIG. 5.—Effect of sulphuric acid on reaction to light. See Fig. 4

described. Groups of tadpoles were placed in tap water in the museum jar, a number of readings were taken at intervals of 1 minute, a small amount of ammonia was added to the water, and the observations were continued for 20 or 30 minutes longer.

Curve 1 of Figure 6 well illustrates the effects of ammonium hydroxide. As may be seen, the group used in this experiment was at first slightly positive. Before introduction of the chemical there were from 13 to 17 animals at the illuminated end of the dish. Ammonium hydroxide had the immediate effect of making the group very strongly positive, the second observation after the addition of the chemical showing 23 animals at the lighted end. Gradually, however, it became less positive, until 4 minutes later it was distinctly negative, with only 6 larvae at the lighted end. This condition also proved to be temporary, and soon the group was positive again. Finally, 20 minutes or so after the addition of ammonia, the number at the illuminated end was in the neighborhood of 20. The group was not only positive but considerably more so than before treatment, and it

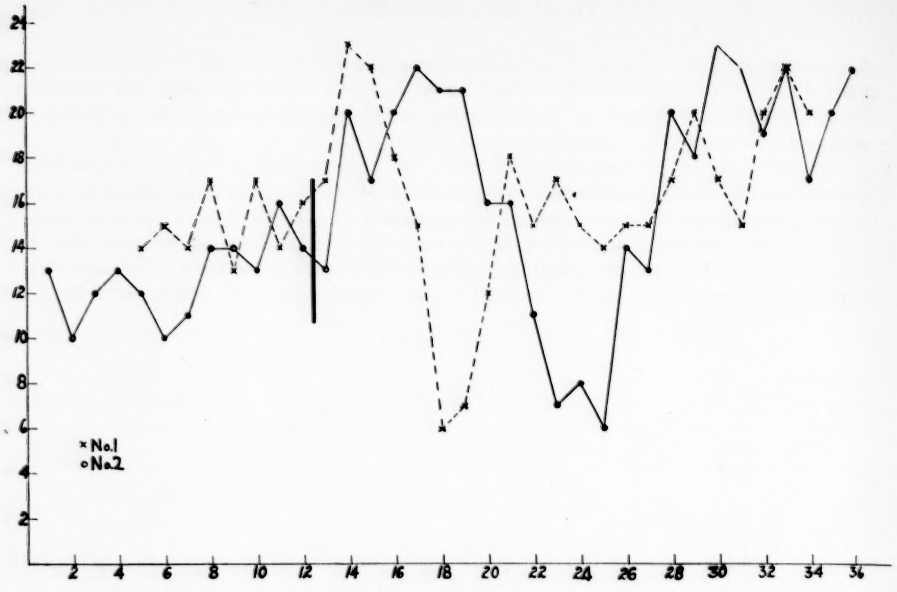

FIG. 6.—Effect of ammonium hydroxide on reaction to light. Constructed as in Fig. 4

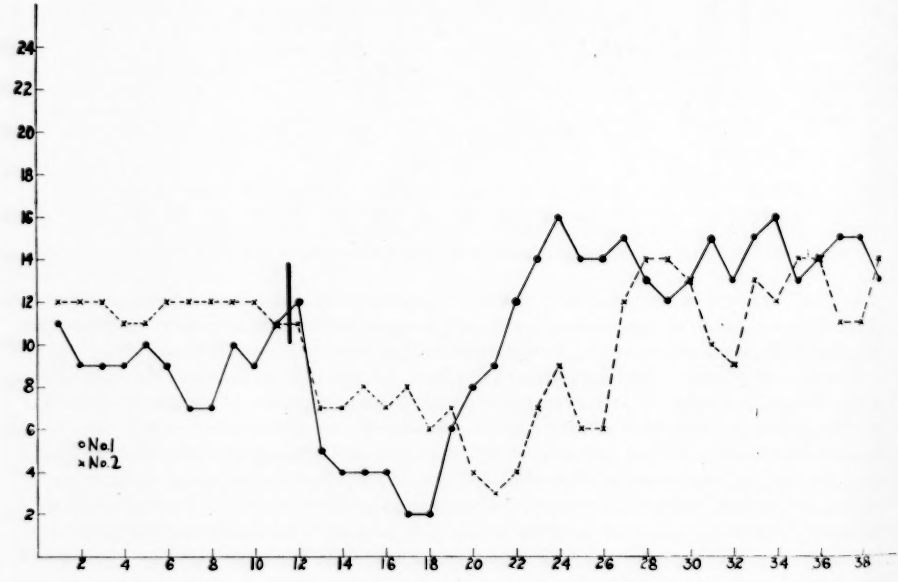

FIG. 7.—Effect of sodium hydroxide on reaction to light. Constructed as in Fig. 4

remained in this condition. Curve 2 illustrates the same general behavior in another group.

Sulphuric acid and ammonium hydroxide thus appear to be opposed in all their effects. The acid causes a marked decrease in positive reaction, followed by an increase, followed, in turn, by a decrease. The alkali causes an increase in positive reaction, followed by a decrease, followed by an increase. The final effect of the acid is to make a group less positive; of the alkali, is to make it more positive.

As may be seen from Figure 7, the effect of sodium hydroxide differs qualitatively, as

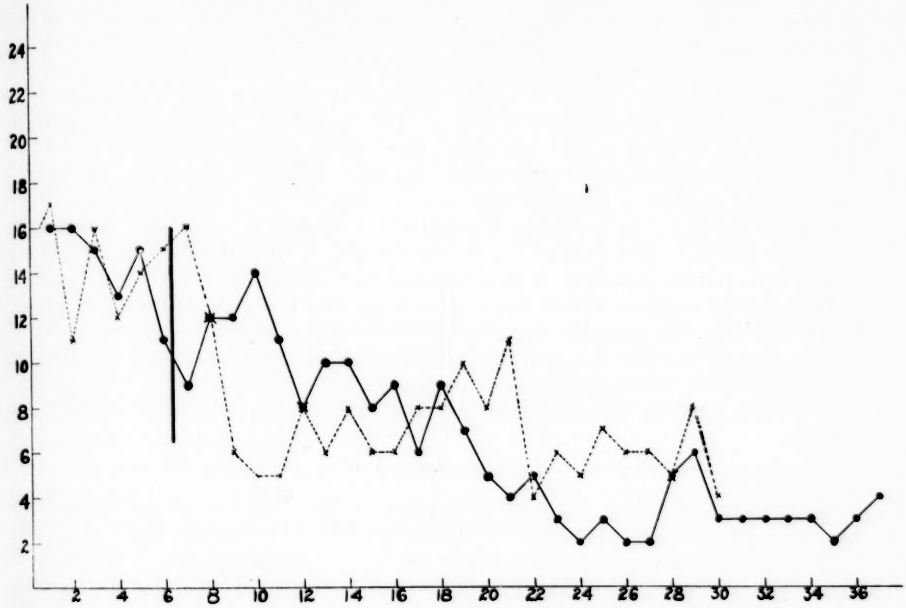

FIG. 8.—Effect of eosin on reaction to light. Constructed as in Fig. 4

well as quantitatively, from that of ammonium hydroxide. The group used in the experiment which is illustrated in curve 1 was, as shown, slightly negative before treatment, with an average of 9 or 10 animals at the illuminated end. Sodium hydroxide had the immediate and decided effect of causing it to become much more negative; within 2 minutes after its introduction the number at the lighted end had dropped to 4, and 3 minutes later to 2. This reduction was followed by an increase, and 13 minutes after the addition of the alkali the number at the lighted end was 14. The group was now slightly positive, and it continued so for the remainder of the experiment.

Curve 2 indicates the same type of behavior. Introduction of the sodium hydroxide brought about a reduction in the number of tadpoles at the lighted end from 11 or 12 to 3, but within 16 minutes the number was back to 12. There was no further change for 24 minutes, when the experiment was discontinued.

From these results, as well as from those previously presented in Table 2, it is apparent that the final effect of sodium hydroxide is not very marked. Half an hour after its introduction the tadpoles exhibit about the same sensitivity to light as before treatment, or, at most, are only slightly more positive. In immediate effect, however, this alkali is almost or quite as effective as sulphuric acid and, like the acid, at first induces the tadpoles to become strongly negative. It differs from the acid in that the strong negative reaction is followed by a return to positive reaction that is permanent.

In view of the very peculiar behavior induced in tadpoles by an acid or alkali, it would seem of interest to investigate in detail the actions of other chemicals, especially of eosin, which, as we have seen, has so pronounced a final effect. The results of two experiments in which observations were made at minute intervals, and in which the tadpoles were treated with eosin, are shown in Figure 8. It is clearly evident from this figure that no devious changes in sensitivity, such as sulphuric acid or ammonia bring about, followed the introduction of the eosin. The reactions of the animals were changed conspicuously, to be sure, but in a direct manner. In both instances the number of animals at the illuminated end of the dish was about 15 before treatment, and in both instances the addition of the eosin resulted in a straightforward, gradual decrease in this number to about 5. The one group required only 3 minutes to bring about this change; the other required 10 minutes or so. As the amount of eosin that was added was not precisely controlled, difference in concentration may account for the variation in time.

If a group of tadpoles is made negative by eosin, they remain negative for at least 24 hours, and they will probably remain so indefinitely if retained in the eosin solution.

The effect of the alcohols appears to be similar to that of eosin, although not nearly so marked. Alcohols simply cause a group to become less and less positive, until finally a minimum is reached. The minimum doubtless depends on the concentration of the alcohol.

DISCUSSION

We have been speaking of groups of tadpoles as being positive or negative but have not explained just what that means with respect to the individuals concerned. According to the scheme adopted, an index of 3 indicates that at any given time there are about three times as many tadpoles at the lighted end of the dish as at the opposite end; but it by no means indicates that three-fourths of the animals are positive and remain indefinitely near the light while one-fourth are negative and stay away from the light. As a matter of fact, there is a continuous interchange of individuals from one end of the dish to the other. A given individual is likely to spend part of the time at one end and part of the time at the other. But if the group to which it belongs is positive, it will probably be near the light most of the time; while, if the group is negative, it is likely to be at the unlighted end of the dish most of the time.

The fact that the reactions to light by tadpoles are influenced by the chemical condition of the medium is not only not surprising but, in view of the findings of numerous other investigators, to be expected. The peculiar behavior induced by acids and alkalies, however, appears to be unique, although Mast (1918) obtained results somewhat similar in the reactions of *Spondylomorum*. He found that these organisms, which were negative at a given intensity of light, could be made positive by treatment with acid, a condition which proved to be temporary, for soon they were negative again. Alkalies had no effect on the reaction.

It is apparent that the experiments with acids and alkalies discussed in this paper are not complete. In every case the experiments were begun with the animals in tap water, which was slightly alkaline. Under this condition, as noted, the final effect of sodium hydroxide was not very noticeable. But it is possible, as there is some evidence for believing, that if, instead of being in alkaline tap water, the tadpoles had been in an acid solution and thus rendered less positive, the final effect of the sodium hydroxide would have been much more pronounced. Any final conclusion, however, awaits further experimentation.

Several theories have been offered to explain the changes in reaction to light that are brought about by chemicals. We have no evidence to show that any of them do or do not apply to tadpoles, except, perhaps, in the case of acids and alkalies. Here the reaction appears to be too complex to permit a simple explanation, such as, for instance, an increase or decrease in permeability. However, the discovery of Allee and Stein (1918), who found that reversal of light reactions in May-fly nymphs was accompanied by change in rate of metabolism, might apply—at least in part. It is certain that the activity of the tadpoles is greatly stimulated, both by sulphuric acid and by the two alkalies that were used; and this might account for the initial reversal. Ordinary observation does not show any further change in activity for some time, although there are changes in reaction to light. However, refined measurements might disclose alterations in metabolic rate during this time.

SUMMARY

1. When a group of tadpoles is placed in tap water in a rectangular aquarium jar, at one end of which a 200-watt lamp is located, the tadpoles usually tend to aggregate at the lighted end of the jar. The group can be said to be positive. Occasionally it is indifferent or even negative.

2. Although the degree of positivity, as indicated by the number of animals at the lighted end of the jar, may vary greatly in different groups, and even in the same group from day to day, it remains constant over a considerable period if the environment remains constant.

3. Various chemicals affect the aggregation of tadpoles in light. If ammonium hydroxide is added to the water and observations made half an hour later, the tadpoles are more positive than they were before the addition. Under the same condition, sodium hydroxide seems to have little effect. Urea probably makes tadpoles slightly less positive. Ether, sucrose, sodium chloride, dextrose, sulphuric acid, and methyl, ethyl, and propyl alcohols make positive groups decidedly less positive.

4. Detailed observations show that sulphuric acid causes tadpoles to become strongly negative a few minutes after its introduction. Soon they become positive again, sometimes as positive as before treatment. Then they become less positive or even negative.

5. Sodium hydroxide causes tadpoles to become strongly negative within a few minutes after its introduction. Later they become positive again, so that the final effect of this alkali is not very marked, even though its immediate effect is very much so.

6. The effects of ammonium hydroxide are opposite to those of sulphuric acid. Upon its introduction the tadpoles become more positive, then within a few minutes much less positive or even negative, after which they become strongly positive again, more so than at the beginning of the experiment.

7. Eosin and the chemicals that were used other than the alkalies and acid appear to have no such involved effects as these. Although they may cause positive groups to become less so, their action is direct.

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SOME FACTORS WHICH AFFECT THE RATE OF MOVEMENT OF THE WINGS IN DROSOPHILA[1]

(Three figures)

LEIGH E. CHADWICK
Biological Laboratories, Harvard University

THE purpose of this paper is to point out certain influences which may alter the wing-rate of an insect and to attempt some evaluation of those which lend themselves to analysis. A thorough knowledge of the sources of variation in wing-rate would enable us to judge not only the nature of insect flight but also the organization of the entire animal much more correctly than is possible at present.

The existence of variation in the wing-rate of a species or of an individual insect was recognized by the earliest workers, Landois (1867) and Marey (1868), and by practically every student who has since dealt experimentally with insect flight. At the same time, there has been a tendency among those who have written of insect wing motion to assume that there exists a definite rate which is natural to each species, and that any variation represents a departure from the normal. Occasionally this assumption is made the basis for false theories of insect flight and insect physiology. The publication of rate measurements, accompanied by only slight indications of the conditions under which they were obtained, has contributed to this situation. It is therefore desirable to show, beyond doubt, that variation is normal, to define the normal limits of variation in one species, and to make a preliminary investigation of some of the underlying causes.

MATERIAL AND METHODS

Specimens of *Drosophila repleta* Wollaston were selected at random from a stock which has maintained itself in one of the animal rooms throughout the year. It is felt that it is of particular interest to give in this first survey some idea of the variation in rate to be found in a more or less natural community, and that efforts to control the factors of heredity and metabolic age may wait until such an exploration has been made. Sex was recorded in each case, although the observations show no correlation with this factor. Size was not measured because it was observed that size is no criterion of absolute wing-rate under the conditions given. The experiments were run during the months of February and March, and no seasonal variation became evident. Altogether, several hundred individuals were used in the experiments.

Wing-rate was measured with an electric stroboscope. The method, which is discussed elsewhere (Chadwick, 1939), consists in tuning an intermittent source of light to synchrony with the wing beat. When flash-frequency and wing-rate are synchronous, the wings appear to stand still, and the rate may be read directly from the dial of the instrument. In order to make such observations of wing motion the specimen must usually be

[1] The writer wishes to express his appreciation to Professors C. T. Brues and A. C. Redfield, of the department of biology, Harvard University, for their continued support and encouragement in this work, and to Professor H. E. Edgerton, of the Massachusetts Institute of Technology, for his generous assistance and advice in technical matters.

placed close to the source of light. It may either be "fixed," i.e., held fast, yet free to move the wings, or it may be confined within a small space. For detailed study of thoracic movements, which are of very small magnitude, only the first of these alternatives is applicable. Rate measurements, however, may also be made, though with more difficulty, on insects made to fly in glass jars or other containers. Some measurements have been possible on larger forms, such as Tipulidae, flying about the room. The rate observations recorded below were made on fixed specimens except where there is a definite statement to the contrary.

Observation of fixed and free specimens of *Drosophila* and other insects satisfy the writer that the wing motion is essentially similar, but not identical, under both conditions. One must always take into account that (1) a fixed insect is an insect with a full load and (2) a fixed insect is nearly always "bothered" by whatever holds him fast.

In many of the experiments it was deemed unavoidable to have the specimens fixed. It was essential that the thorax and legs be left free, first that the motions of these parts might be unhampered and visible, and then because contact of the tarsi with an object usually inhibits flight (Fraenkel, 1932). Small stiff paper points, such as are used for mounting beetles, were tipped with a minute amount of paraffin. The specimen was immobilized with an exposure of 1½–2 minutes to 0° C., tests having indicated that this has no harmful effect; and the paraffined tip was applied under binoculars to the posterior dorsal region of the abdomen and just touched with a hot needle. This doubtless killed the epidermal cells immediately beneath the paraffin (Wigglesworth, 1937); but this result may have been of advantage, in the event that tactile receptors were thereby incapacitated. Care was taken to perform the operation in a similar way on all specimens. Animals whose flight responses were evidently influenced by the nature of the mount were discarded.

For observations on free flight, unmounted specimens were confined in a trough 3 feet by 6 inches by 6 inches, which was illuminated from the ends. Here they showed a positive phototaxis but were not stimulated to fly unless the light intensity was above a definite, though unmeasured, level. The Strobotac with neon bulb was used at one end of the trough. At the other, an ordinary 100-watt bulb, its brightness cut down by ground-glass filters, supplied whatever additional light was needed to raise the total intensity to flight level. The sides and bottom of the trough were lined with red paper, which reduced glaring high lights toward which the insects seemed attracted. A white stripe ½ inch in width was left down the middle of the bottom and appeared to help in keeping the specimens on a relatively straight course. Top and ends of the trough were covered with Cellophane. With this arrangement enough rate readings were obtained to check the results with fixed specimens given below.

It is evident that the general behavior of insects, and hence presumably their wing-rate, is influenced to a large extent by sensory stimuli. For this reason the rate observations on fixed specimens were made regularly with no illumination other than the Strobotron. The intensity of this light varies slightly with the frequency, an unavoidable difficulty; but such variation had no observable effect on the wing motion. Check experiments with varied intensities of illumination were likewise negative. Unnecessary movements were avoided.

The apparatus emits a constant hum of the frequency to which it is tuned, but no response to this or other noises was observed at any time in this species.

Olfactory stimuli were left out of account, it being assumed that the experimental

conditions were reasonably constant in this respect. An attempt to orient freely flying *D. repleta* with the odor of amyl acetate failed.

The existence of a static sense has been looked for regularly but has not been attested in any of the experiments run to date. It is planned to make this question the subject of another communication. The halteres, often mentioned in this connection, must also receive separate treatment. It may be stated definitely that static responses have played no part in the results recorded below.

The responses of *D. repleta* to tactile stimulation during fixed flight are varied. Flight ceases regularly when contact is made with the tarsi, but the tibiae and femora may often be touched with a needle without evoking any response. Touching regions of the abdomen or thorax, or interfering with the wings, may lead to an irregular increase in the wing-rate, followed on continued stimulation by the inhibition of flight and the onset of other responses evidently directed toward evasion. If the head is touched or objects brought near it, this results usually in movements of the forelegs. Flight is inhibited if they succeed in grasping the object. Air currents are also to be classed in the category of tactile stimuli (Fraenkel, 1932; von Buddenbrock and Friedrich, 1932; Hoffmann, 1936) and, since they stimulate flight, may be useful to the investigator. In the present series of experiments, however, all tactile stimuli were avoided in so far as possible. Drafts in the vicinity of the apparatus were shut off by screening. The specimens were not inclosed under glass; this could have been done and would have eliminated chance air currents resulting from movements of the observer, but it also would have made manipulations less convenient and would not have prevented air currents due to movements of the specimens themselves. Flight was induced by picking the mount up suddenly with a pair of forceps, and the specimen was held steady while rate readings were being made. An alternative to this procedure, which has been found useful in photographing flight, has been to fix the mount firmly in such a way that the tarsi of the specimen make contact with the armature of an electromagnet. Closing the circuit depresses the bar, frees the tarsi, and usually results in flight.

After each observation the specimen was returned to the ground on an even keel and released headfirst into an empty vial. Here it remained until needed again.

The observed effects of ordinary sensory stimuli may be summarized by saying that their influence on wing-rate is transient and of small magnitude. Fluctuations from such sources may amount to ± 10 per cent of the basic level (see Table 2, *D*). We presume these fluctuations to be the composite result of the various excitatory and inhibitory stimuli acting on the organism at a given moment, but in practice it is not often possible to analyze the contributory factors separately. The motion of the wings is itself a dynamic phenomenon, entailing tactile and proprioceptive stimulation in the moving parts and metabolic changes in the organism. These considerations are an essential preliminary to any attempt to study the effects of other influences on the wing rate.

THE EFFECT OF FATIGUE

Specimens of *D. repleta*, mounted as described above, were stimulated to fly by means of the tarsal reflex, and the mount fixed in position beneath the binoculars. Flight continued until it ceased spontaneously. Lighting was with the Strobotron only. Rate observations were made at 10-second intervals. Room temperature was constant within $\pm 0°.1$ C. Representative results are plotted in Figure 1.

The longest record obtained was 39 minutes. Records of over 30 minutes are not uncommon. Flights of this duration are hardly to be thought of as part of the natural behavior of the species, and it is therefore rather surprising to find such individuals capable of something in the neighborhood of 486,000 consecutive wing strokes (calculated from Fig. 1, No. 2), especially under fully loaded conditions. In these graphs the more superficial type of rate variation shows to advantage, though it has been smoothed out somewhat by the lapse of 10 seconds between successive readings. The differences and similarities between random individuals are also brought out well. The general downward trend of the curves is inferred to be due to fatigue. At the close of such experiments the specimens could be induced to fly again, but only at low rates and for a few seconds at a time. Generally they survived these flights by only a few hours, while

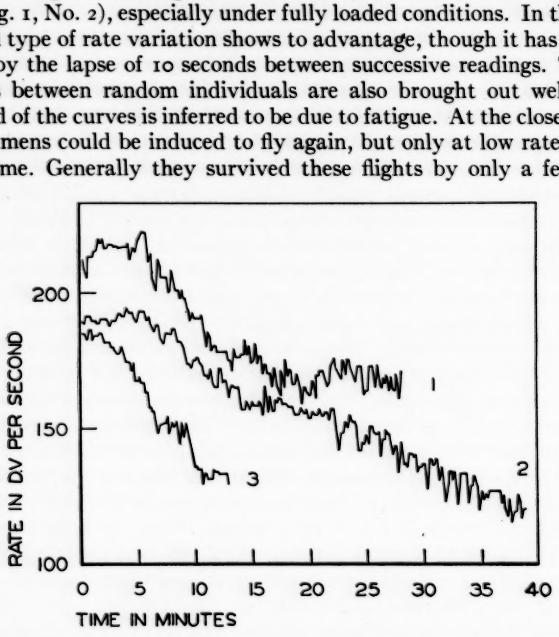

FIG. 1.—Variation in wing-rate of *Drosophila repleta* during continuous fixed flight. Each curve records the flight of a different individual. The flights were made at room temperature, constant within ±0°.1 C., as follows: No. 1, 25°.4 C.; No. 2, 24°.5 C.; No. 3, 24°.8 C.

mounted specimens which had not undergone similar exertion lived upward of 36 hours (unfed) as a rule. The final rate-level lies at about 50–60 per cent of the starting-level in most cases. Marey (1869) states that, when his specimens had become fatigued after a long experiment, the rate fell to a fourth or a fifth of the previous figure. If the insects were then freed, their flight was slow and of short duration. He also found that in fresh specimens the rate might vary by as much as 3:1, and recorded such variation graphically. Magnan (1934) noted a reduction of $33\frac{1}{3}$ per cent in wing-rate during photographic experiments with *Macroglossa stellatarum*, a sphingid, and attributed it to fatigue. Other similar citations could be made if space permitted.

THE EFFECTS OF VARIATION IN ENVIRONMENTAL TEMPERATURE

With few proved exceptions insects are thought generally to be poikilothermic. The observations now to be detailed show that, in *D. repleta* also, wing-rate is very definitely under the influence of environmental temperature. The internal temperature may be

expected to have an even more direct effect, but it has not yet proved feasible to measure the internal temperature of such small insects during rate observations. The rate curves obtained permit the inference that internal temperature in this species parallels external temperature over the greater part of the effective range.

A series of random individuals of *D. repleta* was mounted, as above. The thermometer, graduated to 0°.1 C., was fixed with the bulb 6 inches in front of the Strobotac. Flight was induced by lifting the mount with forceps to the level of the thermometer bulb and about 2 cm. to one side of it. Rate and temperature readings were taken simultaneously during the tenth second of flight. The specimens and apparatus were screened from air currents during the observations. Between 25° C. and the lower limit, temperature was varied by controlling the ventilation of the room. Observations above 25° C. were made by placing specimens and apparatus in a glass-topped wooden case. This was heated, as required, with a battery of electric lights. Armholes in the front of

TABLE 1

ADAPTATION OF *Drosophila repleta* TO CHANGING TEMPERATURE DURING CONTINUOUS FLIGHT
(Specimen No. 9A 3/18/38)

Temperature (° C.)	Wing-Rate (Double Vibrations per Second)	Adaptation Time (Seconds)	ΔT (° C.)
27.3	213	.....	.....
17.4	175	17.0	9.9−
26.8	195	29.0	9.4+
17.6	173	29.0	9.2−
26.4	207	25.8	8.8+
17.6	168	24.2	8.8−
26.1	198	26.6	8.5+

the case allowed the necessary manipulations. The specimens were screened from the heating lights at all times, and the heating unit was turned off during observations. Humidity was not controlled, and excessive dryness may have contributed to the rapid incapacitation of specimens at temperatures slightly above 30° C. Intervals between readings on any one specimen were always longer than 1 minute and were frequently as long as several minutes. Possible artifacts were avoided by using a random, rather than a regular, succession of temperatures.

Since the rate at which the insects might adapt to a change in temperature was not known, experiments were run to test this point. The procedure was as above. Thermometers were placed both inside and outside the heated case. Flight was induced in the usual manner in either situation, and after the first rate reading the specimen was moved, while still in flight, to the atmosphere of different temperature. The length of time required to reach a new steady rate-level was measured with a stop watch. Typical results are recorded in Table 1. The response is reversible and may be reproduced *ad lib* with the same specimen, with results consistent within the usual range of variation. The data quoted show that a specimen in flight becomes adapted to a temperature change of the order of 10° C. within less than 30 seconds. The flight duration of 10 seconds ordinarily employed in the temperature experiments is ample for adaptation to

the small temperature differences between successive observations, a fact which was verified by measurements made after much longer exposures. It is thought likely that the greatly increased rate of respiration during flight (Demoll, 1927; Kalmus, 1929; Jongbloed and Wiersma, 1934) may account for the relatively rapid adaptation noted. It is believed that specimens at rest come to equilibrium with an atmosphere of changed temperature much more slowly.

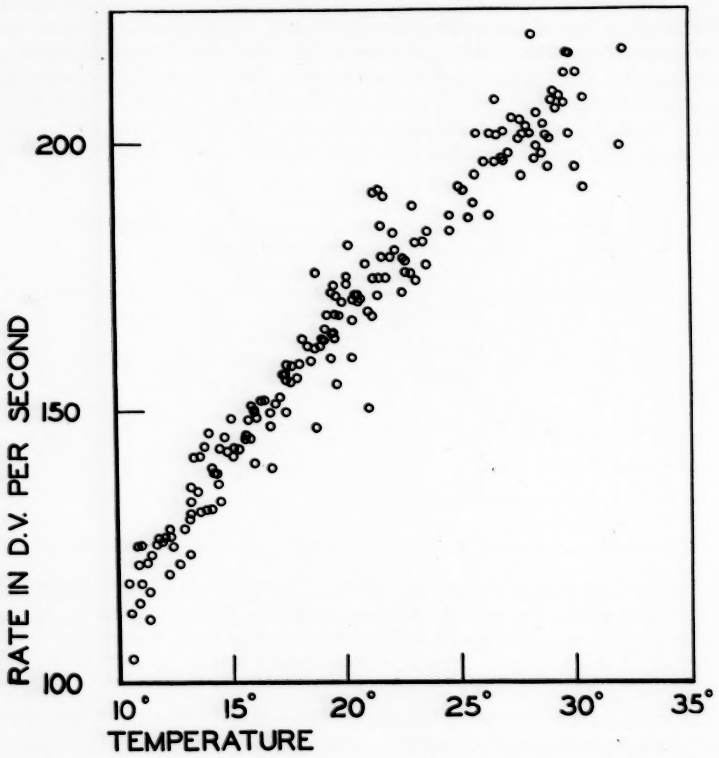

FIG. 2.—Variation in wing-rate of *Drosophila repleta* with varying temperature. Specimen No. 12♂, 2/26–27/38.

A further reason for taking rate measurements only after flight has lasted some seconds is that, during the first few seconds, the rate reaches a value above the steady level presently adopted. This may be a reflex expression of the need for greater energy in the take-off than in maintaining flight. The changes involved follow one another too rapidly for accurate recording with the stroboscopic method but should be analyzable photographically.

The rate-temperature data were plotted for each specimen, as shown, for one example, in Figure 2. Observations were also averaged within 1° intervals (Table 2).

The absolute rate-level may be very different for different individuals (Table 3; cf. also Fig. 1). It is thought likely that metabolic age may be of importance in this connection, but the question remains to be tested. With the random selection of specimens which has been employed, sex and size show no correlation with the absolute rate-level. The slope of the rate-temperature curve is quite constant for all specimens, however; and hence it is considered permissible to average points determined on different indi-

TABLE 2

VARIATION IN WING-RATE OF *Drosophila repleta* WITH VARYING TEMPERATURE*
(Specimen No. 12♂ 2/26–27/38; cf. Figure 2)

T (° C.)	*R* (D.V./Sec.)	*D* (per Cent)	*n*	*T* (° C.)	*R* (D.V./Sec.)	*D* (per Cent)	*n*
10.7	118	11.4	9	22.6	179	5.1	8
11.7	122	8.6	8	23.4	180	2.9	5
12.4	125	4.2	7	24.7	188	2.3	3
13.5	136	9.1	11	25.5	193	4.9	5
14.6	140	6.1	12	26.6	199	5.9	9
15.6	146	4.1	11	27.7	200	3.3	7
16.5	149	5.9	7	28.6	202	8.7	10
17.5	156	4.1	10	29.3	210	3.9	9
18.6	162	9.4	9	30.3	202	5.5	4
19.5	167	6.9	15				
20.5	169	11.0	12	32.2	208	4.4	2
21.5	180	6.7	10				

* *T* = average temperature; *R* = average rate; *D* = greatest deviation from average rate; *n* = number of observations.

TABLE 3

VARIATION IN WING-RATE OF DIFFERENT INDIVIDUALS OF *Drosophila repleta* AT ONE TEMPERATURE LEVEL

Specimen No.	Average Temperature (° C.)	Average Rate (D.V./Sec.)	Number of Observations
12	20.5	169	12
18	20.6	193	4
19	20.5	200	7
1*a*	20.4	174	5
3*b*	20.6	160	7
7*a*	20.5	173	7

viduals in order to construct an average curve for the group. This is rendered necessary because of the impossibility of taking sufficient observations on one individual over the whole temperature range. Figure 3 shows such an average curve plotted from data (Table 4) on twelve individuals.

It should be noted that some real irregularities in the slope of the curve may have been obscured by the process of averaging. The data from numerous individuals agree in suggesting the presence of such irregularities at levels of about 15° C. and 22° C., in addition to the upper and lower limits and the optimum. In different individuals these

irregularities may be displaced slightly along the temperature axis, and therefore tend to disappear in the averages. The individual range of variation at any given temperature, together with the displacement just mentioned, makes it impossible at present to

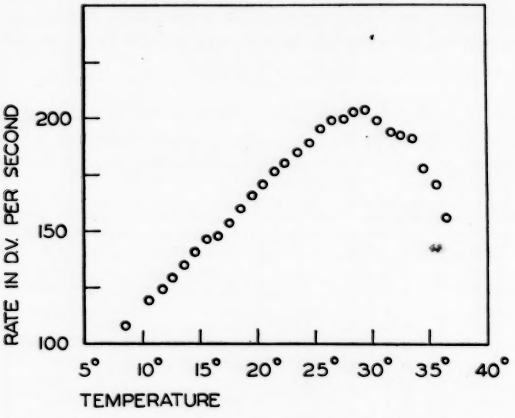

FIG. 3.—Variation in wing-rate of *Drosophila repleta* with varying temperature. The curve is a composite from twelve series of observations. Cf. Table 4 and text.

TABLE 4

VARIATION IN WING-RATE OF *Drosophila repleta* WITH VARYING TEMPERATURE
(Composite from Twelve Series of Observations; cf. Figure 3)

Average Temperature (°C.)	Average Rate (D.V./Sec.)	Number of Observations	Average Temperature (°C.)	Average Rate (D.V./Sec.)	Number of Observations
8.5	108	5	23.6	185	44
10.5	119	15	24.7	189	54
11.8	124	22	25.5	196	19
12.6	129	22	26.5	199	38
13.6	135	28	27.6	200	20
14.6	141	28	28.4	203	25
15.7	147	26	29.4	204	37
16.6	148	32	30.4	199	23
17.6	154	37	31.7	194	9
18.6	160	36	32.5	193	15
19.6	166	62	33.5	192	17
20.5	171	46	34.5	178	10
21.5	177	40	35.6	171	11
22.4	180	42	36.5	156	4

give any statistical confirmation of the existence of these "critical points." But other available facts indicate that it would be unwise to pass them by without further inquiry.

The break at 8°–10° C. is sudden. It has not been possible to induce flight movements at lower temperatures. However, once the starting-level is reached, vibration begins at

a rate of 90–100 per second rather than at zero. This fact strengthens a suspicion that the controlling mechanism lies outside the wing musculature.

The portion of the curve between the lower limit and about 15° C. is all an artifact, in a certain sense. Unmounted specimens could not be made to fly in this range. Only when they had been mounted and jerked into the air did the usual flight-releasing reflex come into play. It is to be doubted that these "flights" below 15° C. have any counterpart in nature. The general behavior below 15° C. is exceptionally docile, as compared with that at higher temperatures. Presumably some far-reaching physiological change takes place near this level.

Again in the neighborhood of 22° C. a less tangible but not dissimilar alteration in behavior occurs. General activity is suddenly heightened. Previously docile specimens all at once become unmanageable. Their power of co-ordination approaches that of more highly organized insects.

Once the optimum temperature has been exceeded, disintegration is rapid. Response is more irregular. One or two flights at 34° C. or above, and often at even lower temperatures, render previously vigorous specimens unfit for further experimentation. They become lethargic and appear unable to spread the wings for flight. Raising these flies from the support induces no more than a feeble effort toward opening the wings, which are, furthermore, carried abnormally and not tucked neatly back as usual. Unlike the changes previously discussed, this reaction is not reversible. Seldom does one of these specimens recover sufficiently, on removal to a lower temperature, to make a flight of even a few seconds' duration. For these reasons the shape of the curve above the optimum has been determined less satisfactorily than below this point.

These observations warn one against too facile an interpretation of the rate-temperature data. Particularly, they suggest that what is being measured here is something which affects the whole organism and not just the flight function, in terms of which the data have been gathered and expressed. At the same time, they justify the expectation that further quantitative studies on wing-rate and related aspects of flight will lead to information of general physiological significance. Should a population be found in which there is less variation in the response at one temperature-level, a favorable opportunity would be presented for the solution of some of the questions raised by this first investigation.

Finally, two additional points of interest may be noted. The average Q_{10} over the range 15°–25° C. is only 1.32. The data, in present form at least, do not fit the Arrhenius equation. In these respects the flight mechanism presents an interesting contrast with what previous workers—for example, Shapley (1920)—have observed in regard to other neuromuscular activities of insects (cf. also Crozier, 1924).

CONCLUSION

The factors for variation in rate which have been noted during these experiments may be classified arbitrarily in two groups on the basis of the magnitude of their effect. Certain factors, such as temperature and fatigue, have a far-reaching and lasting influence, the rate being alterable by as much as 50–60 per cent of its value at, say, 25° C. Other factors, such as sensory stimuli, seem rather to cause temporary fluctuations, within narrower limits, of the basic rate. The level of this basic rate is determined by processes which are unknown but which presumably affect the whole organism and

not only the flight function. It is likely that temperature is one factor which plays an important part in these processes. Fatigue may be supposed to have a more direct effect on the flight mechanism. Species, size, sex, metabolic age, seasonal variation, load, barometric pressure, and humidity are other variables, some of which have already been shown by other workers to have a bearing on wing-rate. Considering the number of these variables and the complexity of the combinations into which they may enter, it is evident that much remains to be done before any satisfactory interpretation of variation in wing-rate can be reached. It would therefore be premature to attempt a comparison of the results reported above with rate-temperature data, such as that accumulated by Crozier (1924), for other neuromuscular activities in insects. At the same time, it is obvious that rates determined without regard to these influences must be largely devoid of physiological significance.

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ACCLIMATIZATION OF FRESH-WATER CILIATES AND FLAGELLATES TO MEDIA OF HIGHER OSMOTIC PRESSURE[1]

(Three figures)

JOHN B. LOEFER
Berea College

THE occurrence of closely related species of Protozoa in fresh and salt water is well known. Whether it be assumed that their ancestral prototype lived in a marine or in a fresh-water environment, one of the diverging groups has obviously become inured to a greatly changed osmotic pressure. Many fresh-water species are known to exhibit a wide tolerance to higher osmotic pressures. For example, Zeulzer (1907) had induced the fresh-water form *Amoeba verrucosa* to live in sea water by gradually increasing the concentration. Chatton and Tellier (1927*a*, 1927*b*) were able to acclimate *Glaucoma piriformis*, *G. scintillans*, *Colpidium colpoda*, *C. steini*, and *Paramecium caudatum* to concentrations of sodium chloride much higher than those in their normal environments. Berger (1929) reported that *Colpidium* was very resistant to changes in osmotic pressure and accordingly was unsuitable for a study of ion antagonism. Viewegerowa (1930) found that *C. colpoda* (?) could adapt itself to a saline solution somewhat less concentrated than sea water. And according to Finley (1930), the following series of forms could be acclimated to sea water: *Amoeba* sp. (*limax* type), *A. verrucosa*, *Bodo uncinatus*, *Colpoda aspera*, *Cyclidium citrullus*, *C. glaucoma*, *Euglena oxyuris*, *E.* sp., *E. terricola*, *Euplotes charon*, *E. patella*, *Lionotus fasciola*, *Monas elongata*, *M. gelatinosa*, *Paramecium aurelia*, *P. caudatum*, *Phacus pleuronectes*, *Pleuromonas jaculans*, *Stylonychia pustulata*, and *Uronema marina*. Frisch (1935, 1937), however, reported that *P. caudatum* and *P. multimicronucleatum* always died when the salt concentration of the medium approached that of 40–45 per cent sea water. Yocom (1934) obtained flourishing cultures of *E. patella* in 65 per cent sea water, although *P. caudatum* and *Spirostomum ambiguum* were less adaptable. Furthermore, marine protozoa can withstand osmotic pressures lower and higher than those of sea water (cf. Schaeffer, 1926). Butts (1935) observed that the marine amoeba *Flabellula* could reproduce in artificial sea water ranging from 20 to 150 per cent, although reproduction in concentrations below 20 per cent was rare. The optimum for growth was 90 per cent sea water, 10 per cent below that of the normal environment. More recently Hopkins (1938) reported that *Flabellula mira* can withstand direct transfer from 100 to 5 per cent sea water, or vice versa.

The extent to which different species possess an osmotic tolerance is of evolutionary significance, for, if acclimatization is demonstrable, the result is a clear case of environmental modification of certain physiological regulatory mechanisms. Tolerance for a higher or lower osmotic pressure may well have been a vital factor in the survival of protozoan species and may at some future time again be vitally important to the species.

[1] Grateful acknowledgment is made to the Kentucky Academy of Sciences for an award for purchase of certain materials and equipment, and to Dr. R. P. Hall for reading the manuscript.

Perhaps it was their degree of osmotic adaptability which originally permitted divergence of Protozoa into salt-water and fresh-water environments.

In order to obtain more information on this problem, which is recurrent in the literature but has received relatively little experimental attention, comparative experiments were undertaken with bacteria-free strains of *Colpidium campylum*, *G. piriformis*, *Euglena gracilis*, *Chlorogonium euchlorum*, and *Astasia* sp. The effects of sudden and of gradual changes to higher salt concentrations were determined, as well as the effects of change from high to lower concentrations.

MATERIAL AND METHODS

Bacteria-free strains of *E. gracilis* and *C. euchlorum* were obtained originally from Professor E. G. Pringsheim, and the strain of *Astasia* sp. was isolated by Dr. T. L. Jahn. The strains of *C. campylum* and *G. piriformis* are the ones used by Elliott (1935) and Johnson (1935), respectively.

Medium "B" (Loefer, 1936) was used for stock cultures. Its composition was as follows: NaCl, 0.003 gm.; $CaSO_4$, 0.015 gm.; $MgSO_4$, 0.0045 gm.; KNO_3, 0.0013 gm.; $FeCl_3$, 0.0002 gm.; distilled water to 1 liter.

The experimental media were prepared by adding Bacto-tryptone (0.2 per cent) and Difco Yeast Extract (0.01 per cent) to Van't Hoff's solution, concentrated (100 per cent) and in various dilutions (1 per cent, 5 per cent, and in successive 5 per cent grades to 95 per cent). The Van't Hoff solution, prepared according to the formula used by Hall (1925), contained 5/8 M solutions of the following salts mixed in the proportions indicated: $CaCl_2$, 1 cc.; $MgCl_2$, 7.8 cc.; $MgSO_4$, 3.8 cc.; KCl, 2.2 cc. Each medium was tubed in 10-cc. amounts and sterilized in the autoclave. Hydrogen-ion concentration in all cases was pH 6.7; incubation was at room temperature (26°–31° C.).

A preliminary experiment was performed in order to determine whether a 1 per cent Van't Hoff medium would support growth of the five species listed above. Inocula of several drops were used for seeding successive subcultures at 3-day intervals. Since all the species were flourishing after nine successive transfers, the medium was considered adequate for maintenance of each species and hence suitable for investigating the effects of different salt concentrations.

The technique used is similar to that employed in earlier investigations (Loefer, 1934, 1935). In carrying out the experiments an effort was made to acclimatize the organisms by allowing them to live for several generations in a given solution and subsequently transferring them to gradually increasing concentrations of saline. Motility, as determined by direct observation of a milliliter sample under a binocular, and viability were the criteria for tolerance.

EXPERIMENTAL RESULTS

SERIES I. *Colpidium campylum*, *Glaucoma piriformis*, *Euglena gracilis*, *Astasia* sp.

Van't Hoff media were made up with salt concentrations of 1, 5, 10, ... , 80 per cent for the first five transfers. In later transfers the concentrations were extended to 100 per cent. After sterilization several tubes at each degree of salinity received a 3-drop inoculum of one of the four species in 24–48-hour cultures in the stock medium. This procedure was repeated for each species. All tubes of the first transfer were incubated at room temperature (26°–31° C.) in moderate light for 24 hours and were then examined

under a binocular for living organisms. If no motile organisms were observed, a viability test was made by transferring 1 cc. of the contents of the experimental tube to a tube of stock medium. The results recorded in Figure 1 designate the highest concentration from which viable organisms were obtained. In all cases the organisms were viable from the lower concentrations tested. Thus, in the first transfer *Astasia* sp. remained viable in all tubes up to and including 80 per cent Van't Hoff solution, *E. gracilis* in all up to and including 35 per cent, and so on.

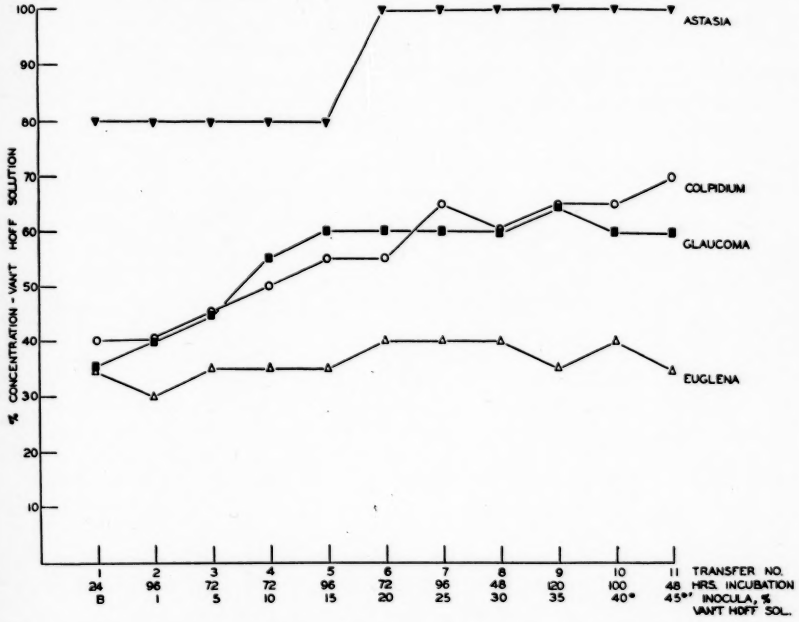

FIG. 1.—Series I. *Astasia* sp., *Colpidium campylum*, *Glaucoma piriformis*, and *Euglena gracilis*. Symbols represent upper limits of viability in each transfer. Asterisk (*) indicates that *E. gracilis* cultures were seeded from a 35 per cent Van't Hoff solution stock; prime (') indicates that *Astasia* cultures were seeded from a 40 per cent Van't Hoff solution stock.

In the second transfer the media of different salt concentrations were inoculated from a first-transfer tube of each species containing 1 per cent Van't Hoff solution. In the third transfer a second-transfer tube, containing 5 per cent salt solution, was used for inoculations. In subsequent transfers the tubes used for inoculation contained 10 per cent, 15 per cent, 20 per cent, ... , 45 per cent salt solution, respectively, the latter concentration being used for the two ciliates in the eleventh transfer. The maximal salt concentrations in which the organisms remained viable in the eleven transfers are indicated in Figure 1, which indicates also the incubation time and the stock medium used for each transfer.

Euglena gracilis showed no definite indication of acclimatization to higher salt con-

centrations, viability being observed only within the range 1–40 per cent; and there was no evidence of an increased tolerance in later transfers. The 40 per cent solutions which they withstood represent a total salinity of 1.59 per cent. The organisms were carried for several subcultures in this concentration, but even this longer period in the near critical concentration seemed to have no effect on tolerance. *Astasia* sp. remained viable in 80 per cent Van't Hoff medium in the first five transfers and in 100 per cent in the last six transfers. Since the higher salt concentrations were not tested in the first five transfers, it was impossible to determine whether this species had shown any definite acclimatization or was merely adaptable to a wide range of salt concentrations. *Colpidium campylum* showed a definite increase in salt tolerance in the later transfers, the limit being increased from 40 per cent in the first and second transfers to 70 per cent in the eleventh transfer. The behavior of *G. piriformis* was comparable to that of *C. campylum*, the former remaining viable in 60 per cent Van't Hoff medium in the eleventh transfer.

Series II. *Colpidium campylum, Glaucoma piriformis, Chlorogonium euchlorum, Astasia* sp.

In this series, which was carried for thirteen to fifteen transfers, the higher, as well as the lower, salt concentrations were used in each transfer. The results are recorded in Figures 2 and 3. It will be noted that the technique differed from that of Series I with respect to the cultures used for inoculation. Thus, in the second transfer of *C. campylum* (Fig. 2), a 20 per cent Van't Hoff culture from the first transfer was used for inocula; in the third transfer, a 25 per cent culture from the second transfer; and in the fifteenth, a 90 per cent culture from the fourteenth transfer. At the last transfer a companion series of tubes (15*A*) was also inoculated from a culture in stock medium as an additional check on the progress of acclimatization. Figure 3 shows the results on the flagellates used in this series. They were carried through thirteen transfers when a companion check series (13*A*) was also incubated. Periods of incubation were identical for the two ciliates, except as indicated in Figure 2 for transfers 15 and 15*A*; incubation times were identical for the two flagellates, as seen in Figure 3. It will be observed from Figures 2 and 3 that viability was noted in salt concentrations higher than those in which the organisms remained motile. This difference was striking in the case of *Astasia* sp., which never remained motile in salt concentrations above 40 per cent.

The results showed acclimatization of *C. campylum* to 95 per cent Van't Hoff solution (3.78 per cent total salinity), of *G. piriformis* to 70 per cent solution (2.78 per cent total salinity), of *C. euchlorum* to 65 per cent solution (2.58 per cent total salinity). These results are significant changes. Thus, on the first transfer from the stock medium, *Colpidium* could not tolerate more than a 30–40 per cent Van't Hoff solution (1.2–1.6 per cent salinity). Acclimatization is also clearly shown by a comparison of transfers 15 and 15*A*. Whereas the acclimated strain of transfer 15 easily tolerated the higher salinities, the stock strain (transfer 15*A*) showed the same osmotic tolerance as at the beginning of the experiment (transfer 1). The negative results with all cultures of the third transfer of *C. campylum* and *G. piriformis* were the result of accidental exposure of the cultures to direct sunlight. In no case did transfer of the ciliates from higher salinities back to the lower salinities result in a loss of viability, and in this respect the results are in complete agreement with those of Series I. *Colpidium campylum*, as well as other species tested, must be subcultured frequently in the media of higher salt concentration.

The limit of motility for *G. piriformis* was a 65 per cent solution; and the limit of

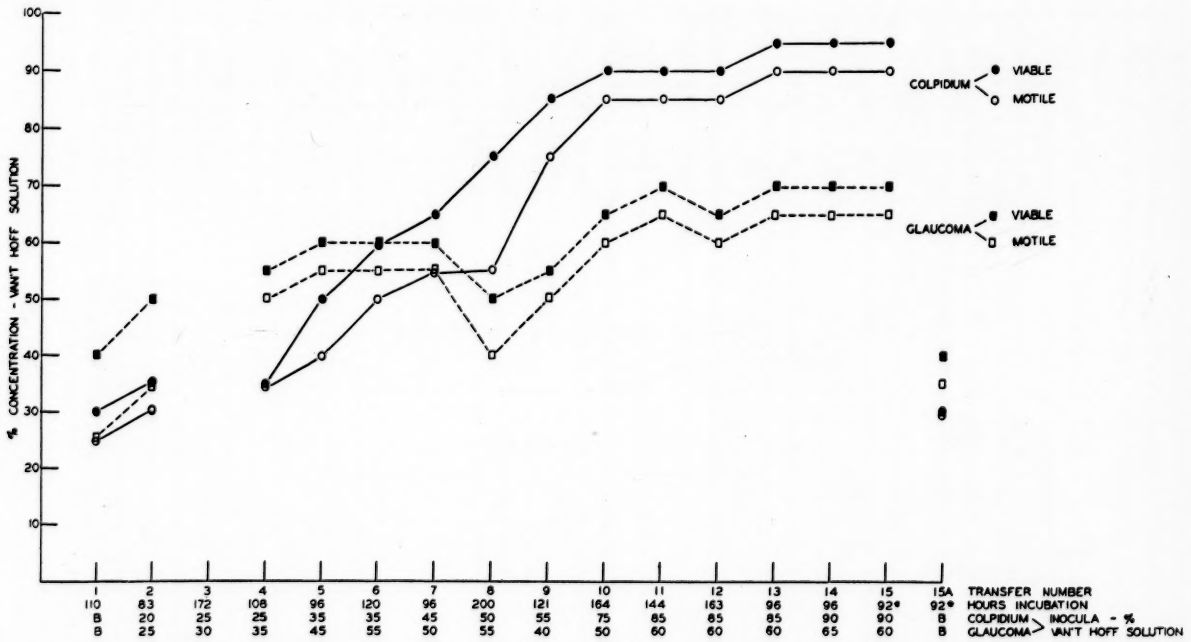
% CONCENTRATION - VANT HOFF SOLUTION
100
90
80
70
60
50
40
30
20
10
COLPIDIUM
VIABLE
MOTILE
GLAUCOMA
VIABLE
MOTILE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 15A TRANSFER NUMBER
110 83 172 108 96 120 96 200 121 164 144 163 96 96 92* 92* HOURS INCUBATION
B 20 25 25 35 35 45 50 55 75 85 85 85 90 90 B COLPIDIUM
B 25 30 35 45 55 50 55 40 50 60 60 60 65 60 B GLAUCOMA
INOCULA - % VAN'T HOFF SOLUTION

FIG. 2.—Series II. *Colpidium campylum* and *Glaucoma piriformis*. Symbols represent upper limits of motility and viability in each transfer. Asterisk (*) = 96 hours for *Glaucoma*.

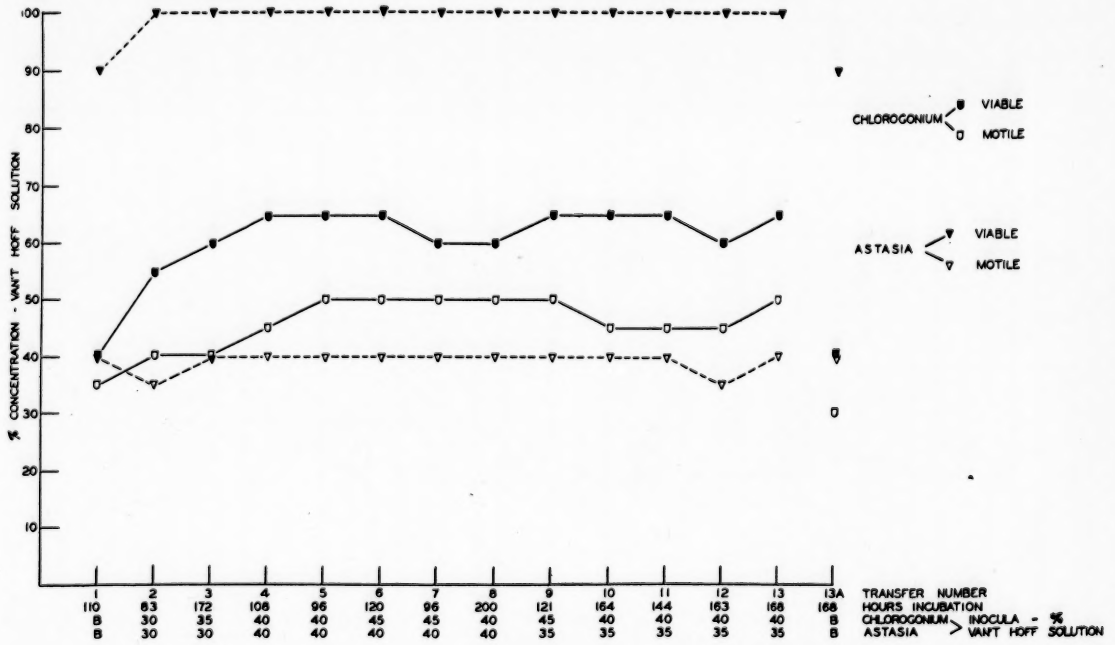
% CONCENTRATION - VANT HOFF SOLUTION
100
90
80
70
60
50
40
30
20
10
CHLOROGONIUM
VIABLE
MOTILE
ASTASIA
VIABLE
MOTILE
1 2 3 4 5 6 7 8 9 10 11 12 13 13A TRANSFER NUMBER
110 63 172 108 96 120 96 200 121 164 144 163 168 168 HOURS INCUBATION
B 30 35 40 40 45 45 40 45 40 40 40 40 B CHLOROGONIUM > INOCULA - %
B 30 30 40 40 40 40 40 35 35 35 35 35 B ASTASIA > VANT HOFF SOLUTION

FIG. 3.—Series II. *Chlorogonium euchlorum* and *Astasia* sp. Symbols represent upper limits of motility and viability in each transfer

viability, a 70 per cent Van't Hoff solution. This species, therefore, withstood a total salinity of about 2.78 per cent after eleven to fifteen transfers. Comparison of transfers 1 and 15*A*, inoculated from cultures in stock media, with transfer 15 indicates the amount of developed osmotic tolerance.

In the first transfer *Chlorogonium* was motile in 35 per cent Van't Hoff solution and viable in 40 per cent solution after 110 hours (Fig. 3); essentially similar results were obtained in transfer 13*A*, also inoculated from a stock culture. In transfers 2–13, however, the organisms withstood greater salinities and in the later transfers survived for as long as 200 hours in a 60 per cent Van't Hoff solution (total salinity of 2.38 per cent). This indicates a definite adaptation to higher osmotic pressures approaching in degree that acquired by *Glaucoma*.

The results obtained with *Astasia* were unusual, since, regardless of the salt concentration of the inoculum, the flagellates were never motile in any of the transfers in concentrations of Van't Hoff solution above 40 per cent (total salinity, 1.59 per cent). In transfers 1 and 13*A* viability was apparently lost upon transfer from cultures in stock medium to 95 per cent and 100 per cent Van't Hoff media, but in all other transfers the flagellates remained viable in 100 per cent Van't Hoff solution. In this respect *Astasia* sp. differs from the other species tested. Furthermore, the increase in tolerance was negligible in the case of *Astasia*, which apparently possesses a very high degree of normal tolerance to high salt concentrations.

Morphological Changes in the Ciliates

The organisms in the solutions with higher salt contents appeared to differ considerably from the original stock. This was especially true of the ciliates, which were no longer recognizable as belonging to the same species. Thus, *Glaucoma* from the 1 per cent dilutions averaged 50 μ in length, but only 42 μ in the 35 per cent solutions. Their contractile vacuoles were no longer apparent in the higher salinities. In concentrations near the vital limit, irregularities in shape, such as may be observed in old cultures, were apparent after several days.

DISCUSSION

From the results described above, it is obvious that *C. euchlorum*, *G. piriformis*, and *C. campylum* gradually developed a tolerance to hypertonic solutions during a series of transfers into more and more concentrated solutions of artificial sea water (Van't Hoff solution). The species differed in the degree of their acclimatization, *Colpidium* having developed a greater osmotic tolerance than the others. The total salinity of the 100 per cent Van't Hoff solution used in these experiments is equal to 3.97 per cent and, therefore, is greater than that of the North Atlantic Ocean, for which Henderson (1927, p. 173) gives a total salinity of 3.2–3.3 per cent. An 85 per cent Van't Hoff solution would contain salts in this concentration. Hence, it is apparent that only *Colpidium* could adapt itself to an osmotic pressure equivalent to that of sea water under the conditions of the experiments. While *Astasia* remained viable in higher salt concentrations, motility was always lost above 40 per cent Van't Hoff solution.

The results on *C. campylum* are in agreement with the general report of Berger (1929). In their experiments with monobacterial cultures of *C. colpoda* and *C. steini*, Chatton and Tellier (1927*a*) found a "distal limit" of 1.0 per cent NaCl, considerably lower than the concentration tolerated by *C. campylum* in these experiments. Finley

(1930) gives further data on *C. colpoda*, indicating a small increased tolerance—from 15 to 20 per cent sea water. It appears that *C. campylum* is more tolerant than either *C. colpoda* or *C. steini*. Viewegerowa (1930) reported also a definite, although temporary, acclimatization of this species. From the rapid division-rate indicated (seven to eight divisions in 24 hours), it seems likely that *C. campylum* or some other fast-growing species was used by Viewegerowa instead of *C. colpoda*.

Glaucoma scintillans, according to Finley (1930), increased its tolerance from 7 to 20 per cent sea water, whereas *G. piriformis* in the present experiments developed a resistance to 70 per cent Van't Hoff solution from a natural tolerance of 40 per cent. As in the genus *Colpidium*, the foregoing appears to be a marked species difference. The highest concentration of NaCl which *G. scintillans* could withstand, according to Chatton and Tellier (1927*a*), was 1.3 per cent; while *G. piriformis* could tolerate 1.8 per cent, a concentration above the vital limit of the other four ciliates they tested. Yet even this is considerably less than the total salt concentration (2.78 per cent) withstood by *G. piriformis* in the present experiments. After the second transfer the ciliate withstood a concentration equivalent to Chatton and Tellier's "distal" limit for this organism.

While the results on *Colpidium* and *Glaucoma* in the present experiments with bacteria-free cultures are not strictly comparable to those of previous investigators, it is interesting to note that the degree of tolerance of these two ciliates resembles that of *C. citrullus*, *C. glaucoma*, *E. charon*, *E. patella*, *L. fasciola*, *P. caudatum*, *P. aurelia*, *S. pustulata*, and *U. marina*, according to Finley's (1930) list of thirty-one ciliated species. Frisch (1935, 1937), however, could not verify the high tolerance of *P. caudatum*. He states (1937): ". . . . many individuals survived until a concentration of 45% was reached." This would be equal to a concentration of about 1.5 per cent saline, which is, however, considerably greater than the 1 per cent "distal limit" for *P. caudatum* reported by Chatton and Tellier (1927*a*).

The adaptability of the genus *Colpidium* to higher osmotic pressures may explain its wide distribution in salt and fresh water. If *Colpidium* is capable of inuring itself to the tonicity of sea water in the course of relatively few generations under experimental conditions, the same thing may have occurred under natural conditions in repeated instances. The generation time of this organism is only a few hours, and the actual number of generations produced in these experiments may be estimated. The total number of incubation hours up to the tenth transfer was 1,106 (1,861 hours through fifteen transfers). If an average generation time of 11 hours is estimated, there would have been 100 generations up to the tenth transfer (169 after fifteen transfers). Environment appears to be the immediate causative factor for this acclimatization; hence, factors of use and disuse or modification of certain physiological regulatory mechanisms are involved, and the inheritance of at least temporarily acquired characters seems clearly demonstrated. The problem confronting the organisms was a vital one, permitting only two alternatives—to develop an osmotic tolerance or die. During the estimated 169 generations most of the organisms no doubt succumbed, although the few that had better regulatory mechanisms lived and multiplied. A similar selective survival method is postulated by Hall and Schoenborn (1939) for the effect of certain nutritive substances on *Euglena*. The duration of these experiments was relatively short; and it is not unreasonable to believe that if *Glaucoma* and *Chlorogonium* had been subjected to finer gradations of tonicity over longer periods of time, they, too, would have shown greater acclimatization. At any rate, they did exhibit an appreciable degree of flexibility in the course of

fifteen transfers. This idea would seem to be at variance with that expressed by Chatton and Tellier (1927*a*, 1927*b*), who contend that it is not necessary to acclimate an organism to higher salt concentration by degrees. Yet it is evident from the present experiments that viability in the higher salinities was not attained unless the stock was already growing in a relatively highly concentrated saline.

Although the tolerance which the flagellates developed was not as great as that of the ciliates, *Chlorogonium* acquired about as much as *Glaucoma*. *Euglena gracilis* apparently acquired none. Although viable in a few instances from a 40 per cent dilution, its motility limit appeared to be 35 per cent. It withstood almost this concentration on sudden transfer. Hence, the salinity tolerated on direct transfer is similar to that given by Finley (1930) for three species of *Euglena*. He found, however, that all three became adapted to solutions of 103 per cent sea water. He states (p. 345): "Non-flagellate motionless stages of Euglenae were frequently seen in the high concentrations of sea water, and many Euglenae were actively swimming in concentrations of 103 per cent sea water." The viabilities recorded for *E. gracilis* up to 40 per cent Van't Hoff solution in Figure 1 were in many cases from nonmotile cultures. Only three of thirteen flagellated species which Finley tested failed to develop tolerance to solutions up to 80 per cent sea water. Hence, the proportion of flagellates which became acclimated is considerably higher than for the ciliates, where twenty-one out of thirty-one developed a tolerance no greater than to 52 per cent sea water. The motility limit of *Astasia* in the present series was 40 per cent, but these organisms were generally viable from all concentrations up to 100 per cent. If we consider viability as a criterion, this unusual organism is euryhaline, but not so if motility is our criterion. Whether the immotile forms represent true cysts is doubtful, since they are not rounded up in the usual manner. Wolff (1927*a*) observed failure of normal encystation of *Hartmanella* in 3–4 per cent NaCl and describes the process simply as a rounding-up. Such a mechanism could conceivably be of great value in the distribution of species, provided they were finally returned to a favorable environment. In fact, it may represent nothing more than a degree of cyst formation.

The unusual behavior just considered is but one of several environmental effects on morphology. There seems to be a diversity of opinion concerning induced morphological changes in protozoan oganisms. As far as these experiments are concerned, the ciliates were most strikingly affected. *Glaucoma* from the 1 per cent solutions averaged 50 μ in length, but only 42 μ in the 35 per cent solutions. Their contractile vacuoles were no longer apparent in the higher tonicities. No slowing of the rate of ciliary movement was observed. These observations are in general agreement with the reports of earlier investigators (Zeulzer, 1907; Wolff, 1927*b;* Kitching, 1934) from their studies dealing with the nature and function of the contractile vacuole. Finley (1930, p. 343) observed in the case of *A. verrucosa* that

> in 84% sea water the contractile vacuole was not present. . . . . The Amoebas were rather active in concentrations as high as 44 per cent sea water, but above that they moved slowly.
>
> The case of the Amoebas was exceptional, for in the other protozoa observed no significant change in morphology occurred until it was apparent that the animal was on the verge of dying. In all the ciliates listed in Table I the pulsation of the contractile vacuole was relatively slower in high concentrations of sea water, but in no case other than *Amoeba verrucosa*, did the vacuole or its pulsation disappear entirely. . . . .
>
> As the salinity increased there was a very noticeable increase in the visibility of locomotor organelles; flagella and cilia were seen with much less difficulty in sea water.

Frisch (1935, 1937) reported decreased size and fewer vacuole pulsations in *Paramecium;* but he interprets these variations as due to decreased food and toxic effects of the salts rather than to direct results of osmotic pressure changes. In the present experiments soluble food for the ciliates was probably present in abundance, considering the general appearance of the controls; yet morphological changes in the higher salt concentrations were appreciable. It might be expected that the contractile vacuole would tend to disappear when its regulatory function became less important, i.e., when the osmotic pressure of the environment approached that of the cell itself. Gaw (1936) observed a much slower pulsation-rate in the contractile vacuoles of certain ciliates when the osmotic pressure of the medium was increased. In accordance with this idea is the finding of Hayes (1930) that the oxygen consumption of *Paramecium* decreased with increasing salinity, which indicates that less energy is required for regulation.

The size differences are of taxonomic interest, for they raise the question of what constitutes a species. Without doubt we had the same organism at the beginning of the experiment; but if the acclimated and normal types of *Glaucoma* had been independently studied from nature, in all probability they would have been considered different species. Such observations make it apparent that consideration must be given to physiological, as well as morphological, details before species can be accurately identified. *Glaucoma piriformis* and *C. campylum* may not be strikingly different in morphology, but they differ considerably in their physiological characteristics. The two forms exhibit different osmotic tolerances, different growth-rates, and different rates of dextrose consumption (Loefer, 1937, 1938*b*). The problem of species delimitation is still, however, a difficult one if organisms exhibit not only physiological but also morphological adaptations when grown in different media. Athanassopoulos (1931) observed such variations in several plankton forms and attributed them to a lessened salinity of the sea water. Morphological changes in the present experiments were, likewise, correlated with salinity changes. Other conditions may cause structural variations; e.g., Lefèvre (1931) reported that changes in hydrogen-ion concentrations caused variation in cellular metabolism as well as in cellular morphology. Changes were also noted and attributed to the hydrogen-ion concentration by Loefer (1938*a*).

The acquired osmotic tolerance of *Colpidium* and *Glaucoma*, however, did not prevent direct successful return to the original medium; and after the change was made, the organisms were again normal in size and appearance after several days. Chatton and Tellier (1927*a*) also observed that *Glaucoma* could be transferred suddenly from a hypertonic to a salt-free medium with no harmful effects, even though it had been growing in a solution near its vital limit for six months. Viewegerowa (1930), too, found that acclimated organisms, when returned to natural medium, soon lost their acquired resistance and in many cases could no longer withstand saline concentrations which were not harmful previously.

SUMMARY

Experiments were performed in an attempt to acclimatize five species of fresh-water protozoa, viz., *C. campylum*, *G. piriformis*, *C. euchlorum*, *E. gracilis*, and *Astasia* sp., to artificial sea water. The culture medium consisted of various dilutions of a Van't Hoff solution to which 0.2 per cent Bacto-tryptone and 0.01 per cent Difco Yeast Extract had been added. After eleven to fifteen transfers, in which stocks from increasingly concentrated salinities were used, *Astasia* was never motile above a 40 per cent dilution; it was

viable, however, even after 200 hours' exposure to concentrated Van't Hoff solution (100 per cent). *Euglena* developed no appreciable tolerance (40 per cent) but withstood 35 per cent on direct transfer. *Chlorogonium* adapted its viability from a normal of 40 to 65 per cent. *Glaucoma*, at first viable from 40 per cent, was finally viable from a 70 per cent solution. The acclimatization of *Colpidium* was greater than any of the other species. Its tolerance increased from a 30 to a 95 per cent Van't Hoff solution. The salinity of the latter is higher than that of ordinary sea water. All organisms, except perhaps *Astasia*, developed their tolerance only gradually through a number of generations. The transfer from the concentrated solutions back to the lower salinities could be made without fatal results.

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A MICROPOPULATION STUDY OF EUGLENA GRACILIS KLEBS IN STERILE, AUTOTROPHIC MEDIA AND IN BACTERIAL SUSPENSIONS[1]

(Five figures)

HELEN E. SWEET
University of Chicago and Santa Barbara State College

I. INTRODUCTION

ROBERTSON'S hypothesis of allelocatalysis has prompted a number of experimentalists to check the relations of numbers of organisms, volumes, and rates of division in order to test his proposition that "there is acceleration of multiplication by the contiguity of a second organism in a restricted volume of nutrient medium" (Robertson, 1924, p. 1242). This may be called the "Robertson effect." Robertson's hypothesis to account for this type of acceleration has been summarized clearly by Allee (1931, p. 166):

> During nuclear division each nucleus retains the charge of autocatalyst with which it was provided, and adds to it during the course of nuclear synthesis. At each division the autocatalyst is shared between the nuclear substance and the surrounding medium in a proportion determined by its relative solubility and by its affinity for chemical substances within the nucleus. The mutually accelerative or allelocatalytic effect of contiguous cells is due to each cell's losing less of the autocatalyst to the medium because of the presence of the other.

Robertson's observations suggest some of the interrelationships existing between members of the same species of relatively asocial animals and plants which are known to affect the structural and functional characteristics of such individuals and even the integration of their communities. Tests of the reality of his theory have been made primarily with protozoans.

The work of several careful experimentalists has given evidence to support Robertson's phenomenon, if not his hypothesis, although even more work with numerous species of protozoans under various conditions tends to uphold the findings of Woodruff (1911), which are exactly opposite. These studies, both for and against the Robertson effect, are thoroughly reviewed by Allee (1931, 1934) and by Johnson (1937). Johnson (1936), whose work with *Oxytricha* and *Paramecium* has included the relations between reproductive rates of different numbers of individuals seeded in different volumes and varying densities of bacterial suspensions, has shown, among other things, that in the presence of sparse suspensions of the bacterium *Pseudomonas fluorescens*, cultures seeded with isolated individuals reproduced faster; with dense suspensions those seeded with two individuals showed greater reproductive rates. Gause (1934) terms this the "Johnson effect" and produces independent evidence in its support.

The relation and interrelation between numbers, food, and volume for each species of

[1] I am gratefully indebted to Dr. W. C. Allee, who suggested this problem to me and whose kindly advice and untiring interest have made the completion of this paper possible after an extended data-finding period and whose help in evaluating and clarifying the results has been invaluable.

animal and plant present in a microcosm is a complex problem, particularly when bacteria and other microörganisms are involved. Johnson (1937) also summarizes much of this work.

These experiments indicate that the number of individuals with which a culture is inoculated is not an absolute factor by means of which the ensuing division-rates of ciliates may be predicted but that with different species, in certain volumes and conditions, seeding a culture with greater numbers insures a faster initial division-rate, and vice versa; the greater numbers generally show relatively greater division-rates in larger volumes and/or when the bacterial content is high.

Such results resemble those which Robertson discovered and assigned to the allelocatalytic effect of a nuclear X-substance given off into the medium during cell division, but these later results can be accounted for in terms of other quantitative relations which exist between the medium and the special organisms studied. There is a recent revival of the original theory in the reports of Karl Reich (1938) with an amoeba, *Mayorella palestinensis*, and of Mast and Pace (1938), with *Chilomonas paramecium*, in which the observed results apparently cannot be attributed wholly to nutrient-organism relations.

Much work has been done on the laboratory ecology of *Euglena gracilis*, which I shall not cite here, in view of the fact that there are extensive reviews and bibliographies to be found in such papers as those of Jahn (1934, 1935*a*), Dusi (1930, 1936), Mainx (1928), Zumstein (1900), and Dangeard (1901).

Since *E. gracilis* is an autotroph, there is opportunity, by limiting the bacteria, to check the effects of numbers of the same species in specific media more closely than with the ciliates or with other heterotrophic organisms.

Experimental work varying the numbers of *E. gracilis* in the inoculum has been attempted only by Jahn (1929, 1930). In a few isolation cultures he found no consistency in division-rates in small volumes. He attempted 2- and 6-drop cultures (size of drop not stated), which were inoculated with single individuals after two washings, details of which are not given. They were followed over a 10-day period. As a result of this experience he concluded that this method was impractical, owing to changes in salt concentrations from evaporation of small volumes over such a period of time and because of surface-interior relations which are not identical with those of cultures with larger volumes.

In mass cultures Jahn (1929) did not obtain absolutely sterile conditions but considered an initial bacterial count of 3,000 per cubic centimeter and a final count of 3,000,000 per cubic centimeter an inappreciable contamination. This number, however, may be responsible, at least in part, for the shape of his growth curves.

With one *Euglena* in from 383,300 to 353,300,000 times its volume of culture media, Jahn found that (1) division-rates decrease as the number of seeded organisms increases; (2) the division-rates decrease as time increases; and (3) the number of organisms increases in time and then decreases. As Jahn says (1930), an increase in numbers, taken alone, is not necessarily a test of an autocatalytic effect in a long-time study (cf. Morgan [1926] and Snell [1929]). Deaths aside, in counts taken at the end of the first 24 hours, however, the division-rate is indicated fairly directly by the number of individuals present.

Jahn (1929) found from mass cultures that the smaller the initial number of organisms per volume, the greater the numbers produced at the end of a 6- or 10-day period. In 48 hours the difference was not great but was slightly in favor of those cul-

tures inoculated with the greatest number of individuals. Without more information one can point out only that this acceleration in the second 24-hour period is what might be expected from either the Robertson or the Johnson effects, so called.

The present paper attempts to explore further the problem of interorganismic effects on the division-rate of *E. gracilis*. Experimental conditions include various number and volume relations, depression-slide cultures, mass flask cultures (not discussed here), relatively sterile cultures employing dilution and irradiation methods, and cultures in controlled bacterial suspensions. The experiments have been made to test the role of numbers of the same species and of controlled populations of associated bacteria with respect to specific volume relations as factors determining division-rates.

Preliminary experiments, using various techniques and types of infusion, showed no consistent results with reference to number in the inocula. These cultures contained such a large number of variables that an attempt was made to set up a series of systems in which the environmental conditions were more nearly under control.

The effects of different numbers in the inocula are mainly investigated, although certain cross-comparisons between growth in different cultures will be given. In this paper the following aspects of the problem are discussed:

1. A comparison of division-rates of cultures inoculated with one, two, four, and eight individuals in several small volumes, using dilution methods for obtaining relatively bacteria-free euglenae.

2. A similar comparison when the euglenae are freed from bacteria by exposure to ultra-violet irradiation.

3. Similar comparisons in suspensions of controlled concentrations of mixed or pure cultures of bacteria when the euglenae are freed from bacteria by both of the foregoing methods.

4. The effects of numbers present on survival after long exposure to ultra-violet irradiation.

5. A comparison of the results with these small-volume cultures with those of Jahn's mass cultures.

II. CULTURE METHODS

A clone of *E. gracilis* was isolated at the beginning of the summer of 1934 from a culture obtained from the General Biological Supply House, Chicago. The first clone served for all the cultures of 1934 and 1935. A second clone, established in the spring of 1936 and used for work of that summer, was found to be no different statistically in division-rates or any other measured characteristic from Clone I. This clone was isolated from a culture furnished by the Albert Galigher Laboratories of Berkeley, California.

The standard experimental medium throughout was the salt solution of Osterhout (1906), as modified by Barker and Taylor (1931), made with block-tin and then glass-distilled water and using analyzed grades of the following salts in a liter of water: 7.8 gm. $MgCl_2 \cdot H_2O$; 3.8 gm. $MgSO_4 \cdot 7H_2O$; 2.2 gm. KCl; 1.0 gm. $CaCl_2$; and 1.0 gm. NaCl. This was diluted 500 times with double-distilled water before autoclaving and using. One cubic centimeter of M/20 NaH_2PO_4 was used to buffer each 30 cc. of the distilled solution, and pH adjustments were made with M/20 NaOH.

Ferric chloride was added to each liter of the salt solution before using it, so that there was a concentration of 0.00002 per cent ferric chloride per liter. Unlike usual practice in flagellate culturing, no acetate, citrate salts, or organic material was used, since a medium

containing as little organic matter as possible was desired. Glucose, Leibig's extract, Difco tryptophane, peptone, or muscle extracts are added by most recent experimentalists, such as Jahn (1929–35), Alexander (1931), Hall (1933), and Elliott (1938). While several students of chlorophyll-bearing organisms, such as Zumstein (1900) and Ternetz (1912), reported unfavorable growth in an autotrophic medium, Pringsheim (1912) and Mainx (1928), working with both heterotrophic and autotrophic media, conclude that growth of flagellates was as good in an autotrophic as in a heterotrophic medium. They do not, however, present evidence concerning the degree of bacteriological sterility of their media. Jahn used the inorganic salt media of Mainx (1928) and Gunther (1928), to which he added hydrolyzed casein for most of his work. This salt solution also had either ammonium or potassium nitrate salts as nitrogen sources. Dusi (1933) succeeded in culturing several species of *Euglena*, using inorganic sources of nitrogen, and later (1936, 1937) carried through four transfers, at least, in a totally inorganic medium. Dusi also finds that growth is much facilitated when organic materials are present. Mast, Pace, and Mast (1936) report success in growing *Chilomonas*, a supposed saprozoite, on an inorganic medium, while Hall and Loefer (1936) failed to grow this species when the dilution of tryptone was beyond 1:100,000.

The solution used in the present work lacks nitrogen in quantities determinable by sulphanilic nitrite or Nessler's ammonium tests. However, the reagents used have minute quantities of nitrogen present as impurities; and, likewise, the distilled water shows traces of it. The question of nitrogen-fixation on the part of *Euglena* is entirely problematical. There was no evidence from tests that the nitrogen content increased in cultures in sterile salt solution in a 24-hour period. It is possible that sufficient nitrogen is stored within each organism to supply several generations, or the disintegration of one or more of the original individuals in a culture may furnish enough of this substance. The amount of organic stuff in a 1:500,000 dilution of the stock medium must have been an extremely meager source of nitrogen or other essential organic matter. This lack of available nitrogen may account for slower division-rates in cultures started with one or two individuals in relatively large volumes when compared with those given with larger numbers in the inoculum.

The present results concur with those of Dusi (1937), Hutner (1936), Jahn (1935*a*), Hall and Schoenborn (1939), and others, in that growth was not as abundant or as assured in an inorganic medium as in infusions of several types. Cessation, and finally death, was a common result from cultures in salt solutions in large volumes. This inability of cultures to continue growth regularly in sterile inorganic salt media or to attain maximal numbers in mass cultures in such media, even with bacteria present, undoubtedly is reflected in the early losses in isolation cultures.

Stock cultures grew abundantly in wheat infusions which were made by boiling 10 grains of dried wheat in 100 cc. of distilled water for 10 minutes and allowing it to stand 24 hours before being inoculated with several drops from an established culture. Ten thousand *Euglena* per pipette drop (0.05 cc.)[2] was a typical count in such infusions.

The original stock culture was developed from an isolated individual which produced twelve euglenae in 24 hours. The division-rates of subclones were carefully studied to detect possible variation in cultures with small volumes. In wheat infusions, where bacterial contamination and evaporation are great, no variation in division-rates, which

[2] Unless specifically stated to the contrary, the drops used in this study contained approximately 0.05 cc.

were statistically significant, appeared in 26 hours. In 36, 42, and 66 hours significant variations did occur. Readings at the end of 24 hours only are used in this paper; hence no significant variation is to be expected as a result of differences inherent in the euglenae themselves.

In preparation of any series for an experiment the following dilution methods were employed to reduce the number of bacteria. This method was used for all experiments unless otherwise designated as sterilized by exposure to ultra-violet irradiation, as described at the beginning of Section IV.

One drop of a stock culture, approximately 2 weeks old, was mixed with 5 cc. of autoclaved salt solution in a sterile watch glass[3] and was allowed to stand 1 hour. This is called "dilution Bath A": it diluted the stock 101 times. Bath B was made by transferring 1 drop of Bath A to 10 drops of sterile salt solution, diluting the stock 1,111 times. Euglenae were kept in this bath 10 minutes. Bath C involved a transfer of 5 micropipette drops (0.0025 cc.), each microdrop containing one *Euglena*, to 5 large drops (0.05 cc.); and this mixture was allowed to stand 20 minutes. This last bath brought the total dilution of the stock medium to 23,331, and the concentration of the stock became approximately 0.00004.

As euglenae were transferred from Bath C to sterile salt-solution cultures of from 1 to 40 drops, the cases of greatest concentration of the stock present in any of the experiments would occur with eight euglenae present in 1 drop. This further dilution would contain approximately 0.000002 concentration of the stock materials of bacteria.

These changes in concentration were gradual enough to avoid shock, since there was no readily apparent change in motility or morphology.

Bacterial counts (Table 1), employing plating methods as used in standard water analyses, were taken at various intervals in the dilution series. Counts made at the end of the first 8 minutes in Bath C showed fewer bacteria in the salt solution than at the end of 10 or 12 minutes, after which time there was no increase. Tests made at the end of 15 minutes in Bath C, according to standard bacteriological methods, rarely showed more than seven viable bacteria present per cubic centimeter. Later in the present paper, when cultures are said to be "sterile" or "bacteria-free," this degree of sterility is meant.

Stock cultures tended to become alkaline, pH 8.1–8.5, whereas the sterile salt solution was buffered to a pH of 7.6. Subcultures in small drops kept the same pH at least 24 hours. The change from stock-culture media of greater alkalinity apparently offered no great physiological hazard, although this may have been one of the reasons that reproductive rates were greatest in wheat solutions.

Because of the use of carbon dioxide by autotrophs, carbonic acid does not accumulate. If CO_2 utilization is a measure of division-rate or of metabolism, the more alkaline media might indicate more complete metabolism; and, likewise, greater acidity might be associated with less complete use of materials in the medium and with comparatively slower division-rates.

Quantitative studies on the optimal pH for the growth of *E. gracilis* in various types of organic media and with various temperature and light relationships have been reported by Kostir (1921), Jahn (1931, 1932, 1935*a*), Hall (1933), Wang (1928), and others.

Measurement of CO_2 consumption, O_2 production, and oxidation-reduction potentials

[3] All glassware was washed with soap and water and rinsed with flowing hot tap water or was allowed to stand in glass-cleaning solution overnight and rinsed thoroughly with running hot tap water and dried with clean linen towels, wrapped in heavy tissue paper and sterilized in a dry oven at 190° C.

for *Euglena* species have not been reported. Jahn (1933) found, with *Chilomonas*, that the reduction of sulphur was very important in establishing high division-rates. Certainly, CO_2 sources are of great importance to a chlorophyll-energy system. Because

TABLE 1

NUMBERS OF COLONIES OF BACTERIA IN 1 CC. OF VARIOUS SALT-SOLUTION BATHS AND CULTURES OF *Euglena gracilis* EMPLOYING THE AGAR-PLATE COUNTING METHOD

(Average of ten cases, 1935 and 1936)

Section	Culture	Time Elapsed	Mean	Standard Deviation
A*	Stock	2 wk.	4,330,000.0	500,400.0
	Bath A	60 min.	34,200.0	8,600.0
	Bath B	10 min.	4,600.0	1,200.0
	Bath B	20 min.	8,300.0	2,300.0
	Bath C	8 min.	2.0	1.0
	Bath C	10 min.	3.4	1.0
	Bath C	12 min.	3.3	1.0
	Bath C	15 min.	3.0	0.1
B†	*a*) Washed sterile cultures in sterile salt solution (8 euglenae present)	10 min.	1.00	0.05
		24 hr.	0.75	0.01
	b) Ultra-violet radiated 2-minute salt-solution culture (8 euglenae present)	10 min.	1.20	0.04
		24 hr.	0.06	0.00
	c) Ultra-violet radiated 3-minute salt-solution culture	10 min.	0.00	0.00
		24 hr.	0.01	0.02
C‡	*a*) X concentration No euglenae present	10 min.	3,109,700,100	75,000
		24 hr.	3,107,900,100	49,100
	8 euglenae present	10 min.	3,100,600,060	54,200
		24 hr.	3,111,637,940	71,230
	b) 0.001 X concentration No euglenae present	10 min.	2,876,000	4,920
		24 hr.	2,500,900	5,700
	8 euglenae present	10 min.	2,914,912	7,200
		24 hr.	3,241,617	7,140

* Bacterial counts from dilution baths in preparation for washed-sterile euglenae cultures.

† Bacterial counts from 1 drop of washed-sterile, irradiated 2-minute, and irradiated 3-minute cultures with eight euglenae present at the beginning and end of an experiment.

‡ Bacterial counts from *Pseudomonas* suspensions of X and 0.001 X concentrations with and without euglenae present.

division-rate is notably higher in organic media, it is easily surmised that a major function of certain bacteria or of organic radicals may be that of serving as links in the carbon or nitrogen cycles of *Euglena* metabolism.

Temperature has been constant to within 0°.5 C. in any one series of experiments.

Temperatures of 23°, 25°, and 27° C. have yielded approximately similar results in 1935, 1934, and 1936, respectively; at least, no significant differences could be found in the average division-rates shown by comparable cultures in these three series.

Constancy of light, humidity, and temperature were maintained by use of a specially constructed chamber, which was operated in a room with controlled temperature and humidity. This chamber, designed by Dr. W. C. Allee, is a cylinder 3 feet in diameter and 3 feet in height with a revolving press-board floor. It had as its source of light six 100-watt "daylight" mazda bulbs, each with a reflector and suspended from an overhanging disk 3 feet above the floor of the chamber. The light passed through a heat screen of over 2 inches of filtered-water plate glass and caused little distortion of the light. During an experiment the chamber was lighted constantly, day and night. The thermograph records of the temperature within the chamber showed the variation to be no greater than 0°.5 C. The humidity of the constant-temperature room was under positive control and remained around 67 ± 2 per cent; within the light chamber there was less variation from this mean. It was also possible to place the crystallization dishes holding the depression slides of an experiment in this chamber without any movement of the cultures within a given experimental period.

III. A COMPARISON OF NUMBERS PRODUCED IN CULTURES INOCULATED WITH ONE TWO, FOUR, AND EIGHT INDIVIDUALS IN VARIOUS SMALL VOLUMES

Cultures of *Euglena* (washed free from bacteria, as described in the previous section) showed certain similarities in division-rates dependent on the number of organisms which were placed in a given volume. As always in these experiments, all euglenae were from the same clone. In these particular tests they were cultured in from 1 to 40 drops of fresh sterile medium. Series were arranged so that there were eight individuals isolated singly in eight different depression slides; four slides were inoculated with two organisms each; two slides similarly were seeded with four and two others with eight individuals each. One of the last served as a control on which bacteriological tests were made.

This set of sixteen slides constituted one experimental series. Forty individuals were required to inoculate such a series. Each organism was equally and separately washed and was finally transferred to its respective slide within a half-hour of other members of the series. Each slide was covered with a thin cover slip, which was held in place by a film of vaseline or sterile water. Evaporation was reduced to a minimum by placing the covered slides in a moist culture chamber composed of a large crystallization dish in the bottom of which was about an inch of water; the slides were placed on a white platform above the water. Such a chamber, holding eight depression slides, was transferred to the constant light, temperature, and humidity chamber already described. The two crystallization dishes which housed a single experimental series were kept close together, so that all slides received approximately equal illumination.

The subcultures were left undisturbed until they were removed to be counted at the end of 24 hours. The control slide was tested bacteriologically by the plating method. Contamination could not be determined until another day had elapsed; hence, no experiment was concluded until at least 52 hours after it began. Twenty-four-hour counts of *Euglena* proved most satisfactory; in 48 hours there was evidence of slight evaporation, even though the slides had not been inspected in the meantime.

Depression slides were used for volumes of from 1 to 5 drops; watch glasses were used

for those of 10, 20, and 40 drops. The latter were covered with heavy glass slides made to fit and sealed with vaseline. Methods of sterilizing all glassware were described in Section II of this paper.

The data accumulated in three years of experimentation have been summarized in Figure 1 since there were no significant differences in the results for any one year which could make invalid a treatment of the data as a whole. The graphs plot the average total

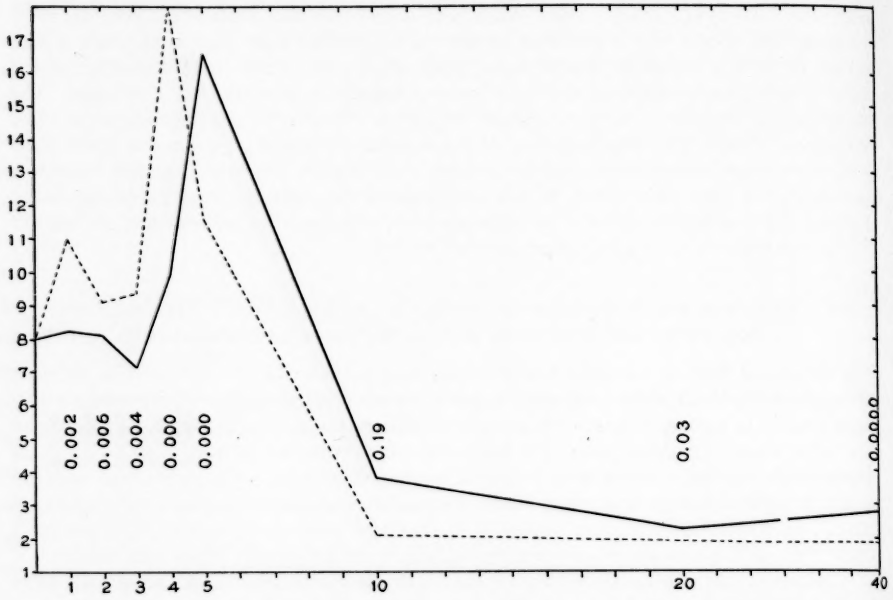

FIG. 1.—Average total numbers of *Euglena gracilis* present after 24 hours in cultures inoculated with one and two (dotted line) contrasted with four and eight (solid line) individuals with statistical probability of the differences. Ordinates, average total numbers present after 24 hours; abscissas, volumes in drops. Numerals give the statistical probability (P) of the observed differences at the several points. This was determined by Student's method for paired experiments. $P = 0.03$ means that there are three chances in one hundred of finding as great differences by random sampling. $P = 0.05$ is usually considered to be the extreme upper limit of statistical significance. The smaller the value of P, the greater the significance.

number of individuals produced in 24 hours in cultures started with one and two euglenae (broken line) and those started with four and eight (solid line) in the various volumes. In addition, the statistical probability of the differences are given for each volume.

Table 2 shows the number of series which have been summarized in Figure 1. The fully expanded table, showing comparisons for the different volumes for the experiments conducted in 1934, 1935, and 1936, summarized by years, is given in detail elsewhere (Sweet, 1937). The summary in Table 2, together with the statistical probabilities shown on the figure itself, gives some indication of the reality of the recorded differences. The

fact that essentially similar results were obtained in three different periods of experimentation adds to their reliability.

In the smaller volumes the cultures started with fewer euglenae reproduce faster than those seeded with four or eight individuals. The mean difference in the average total numbers in the cultures inoculated with one and two euglenae is greater than in those having four and eight in the inocula in 1, 2, 3, and 4 drops; the actual difference is 2.92, with $P = 0.00001$. In 4 drops taken alone, the same cultures also reproduce more rapidly; the difference is 9.5 with $P = 0.0000$.

In 5 drops conditions are reversed; the difference is 6.26 with $P = 0.0000$. In the largest volumes tested, 20 and 40 drops, the average total numbers maintained by the two groups show statistically significant advantage for the larger number in the inocula, with a difference of 1.00 and $P = 0.0000$.

There is very little difference, none of statistical significance, in the average division-

TABLE 2

A SUMMARY SHOWING THE AMOUNT OF EXPERIMENTATION USED AS A BASIS FOR FIGURE 1

Culture Volume (in Drops)	Number of Series		
	1934	1935	1936
1	16	16	9
2	16	15	.....
3	13	13	13
4	10	3	13
5	8	12	.....
10	.....	8	.....
20	.....	11	7
40	.....	15	9

rates of each group in the three smallest volumes of 1, 2, and 3 drops, in spite of the fact that the volumes are doubled and tripled. There is, for example, no significant advantage or difference in the numbers produced in cultures started with a single individual in 1 drop, as compared with 2 or with 3 drops of culture medium.

As seen in Figure 1, decided differences in division-rate appear at the transition from 3- to 4-drop cultures; the average rates are doubled, or practically so, for the cultures inoculated with one and two organisms; and there is a 50 per cent acceleration for those started with four and eight individuals. Cultures with one and two euglenae in the inocula attain the highest rates of reproduction to appear anywhere in the series tested. The acceleration in cultures with least numbers in the inoculum, as compared with those with greater, a phenomenon which is typical of the smaller volumes, is greatly enhanced in 4 drops. This volume of 4 drops represents the optimal volume for seedings of one and two euglenae.

In 5 drops the cultures inoculated with the greater numbers show significantly higher reproductive rates, and these reach their maximum in 5 drops. Cultures seeded with one and two individuals decline in division-rates from cultures of 4 drops to those of the 5 drops almost as rapidly as they increased from the smallest volumes to those of 4 drops.

In 4 drops the cultures seeded with eight euglenae had least numbers at the end of 24 hours; in 5 drops these cultures increased the most and attained a maximal number for all volumes tested of those seeded with eight organisms. The optimal volume of cultures inoculated with four and eight individuals is, under the conditions of these experiments, 5 drops; whereas, cultures seeded with one and two individuals reached their maximal numbers in the 4 drops.

In the twenty series tested in 5 drops the cultures inoculated with four and eight individuals gave an average total number of 16.88 at the end of the first 24 hours; similar cultures started with one and two euglenae produced an average of 10.62. The chance of this occurring in random sampling is less than once in several thousand times, $P = 0.0000$.

When identical volume relations obtain per individual in the inoculum and the numbers produced are compared, the cultures started in 5 drops show significantly higher rates than do those of either supraoptimal or suboptimal volumes. One comparison was possible with the suboptimal volumes, and six with the supraoptimal. There were seven cases in which cultures of 4 drops could be contrasted with suboptimal volumes, in which identical volume-per-individual relations existed; and of these, five gave significantly more euglenae in the larger volume. One of the two cases in which the cultures in 4 drops could be compared with supraoptimal volumes showed significantly more organisms produced in 4 drops.

In brief, from this type of comparison it is clear that it is not only the relationship of volume per individual which acts to account for differences in division-rates, but there is, under the conditions of these experiments, a volume which tends to be optimal because it is conducive to greater division-rates almost regardless of the number of inoculated euglenae or initial volume-per-capita relationships.

In 10, 20, and 40 drops (Fig. 1), which constitute the three largest volumes tested, the division-rates fall for all cultures, irrespective of size of inoculum; maintenance of original numbers is impossible; and the cultures seeded with four and eight individuals show significantly greater ability to survive.

In 10 drops considered alone, the cultures with eight initial individuals show significantly greater numbers than do any other cultures; the probability of these differences[4] is in the cases of 1/8 $P = 0.004$, 2/8 $P = 0.0000$, and 4/8 $P = 0.004$. The cultures with four and eight individuals in the inocula, when grouped as in Figure 1, however, do not show significant advantage over those with one and two euglenae seeding a culture in this supraoptimal volume.

In 20 drops there is a slightly significant difference in the division-survival rates of the cultures; four and eight organisms in the inocula make the better showing ($P = 0.03$). In 40 drops cultures with four and eight in the inocula, in 24 parallel cases, show a very reduced but a significantly greater number present after 24 hours than do cultures started with but one or two euglenae.

With the suboptimal volumes one can postulate that one or more of the following factors are acting: (1) too great concentration of excretory products; (2) too great concentration of a growth-promoting substance or substances; (3) too great evaporation and hence a too rapid or too great change in salt concentration or too concentrated an

[4] "1/8" refers to a contrast between the division-rate of the cultures inoculated with one organism and those inoculated with eight. The statistical significance of the difference is given as P.

end-product; (4) too great an intake or accumulation of oxygen and far too rapid a loss of carbon dioxide; and (5) too few bacteria or bacterial end-products.

With the supraoptimal volumes the converse of these same conditions holds as possible responsible agents to account for the harmful effects of the greater volumes: (1) too much toxic material per organism; (2) too great dilution of growth-promoting substance or substances; (3) too slow loss of carbon dioxide; (4) too slow intake of oxygen; (5) too slow evaporation, i.e., too slow a change in osmotic pressure; and (6) too many bacteria or bacterial end-products.

In view of the instances in which equal volume-per-organism relations have shown that volume per individual is not, by itself, controlling the division-rates (as explained on p. 182), it is likely that those factors connected with surface-volume differences are those most active in altering the numbers produced or surviving. Points (3) and (4) of both foregoing lists and (5) of the second list are at once subject to surface-volume differences. It is easy to see that in the suboptimal volumes evaporation may play an important role and that, by change in the osmotic pressure of the media, may be deterring division. This is probably the major cause of depression in suboptimal volumes.

On the supraoptimal side of the curve the causal relations are less clear. At present we can only suggest that the relatively increased mass and relatively decreased surface may be associated with the depression, or that the medium is toxic and the toxicity shows up most readily when there is a large volume per individual. This well-established mass effect is discussed at some length by Allee (1931, 1938). As might be expected from similar studies, the toxic effect was less pronounced in cultures seeded with the greater number of euglenae, notably in 10, 20, and 40 drops. Although the average population for such cultures always decreased, differential survival was exhibited in favor of increased numbers in the inocula.

The greatest numbers produced in the small-volume, suboptimal cultures, when contrasted to the numbers maintained in the supraoptimal cultures, is evidence that the conditions obtaining in these small volumes approach the optimal more nearly than do those in the larger volumes for the size of inocula used.

The phenomenon which appears most dramatic in this survey of division-rates of washed-sterile euglenae is the rapid reversal of the size of the inocula which yields maximal numbers. Four and 5 drops represent the optimal volumes for division-rates of euglenae in this type of culture. With the addition of 1 drop of culture media the optimal number of euglenae seeding cultures was changed from the smaller to the greater number in the inocula. It is not clear why this reversal should occur so rapidly and in these particular volumes. However, such a reversal is in line with the experience of certain workers with similar relationships for ciliates, notably Peterson (1929) and Johnson (1933, 1936), except that with their work the transition was less sudden.

Evidence of the Robertson effect or of some modification of it is present in the cultures in 5 drops of these salt-solution series which are relatively free from bacteria. Reproduction and/or survival rates "are accelerated by the presence of contiguous organisms in a limited volume." In the supraoptimal volumes, of 10, 20, and 40 drops, there is certainly a loss of individuals and probably a diminished division-rate in the survivors; even so, more individuals are present in cultures inoculated with greater numbers.

There is no evidence that this effect, even in 5 drops, is to be accounted for by "allelocatalysis" as explained by Robertson. The contribution of nitrogen to the media, made possible by the death of one or more individuals in the larger volumes inoculated

with greater numbers, might account for the observed effect. Also, because of the fact that volume per organism is not in all cases a controlling factor, there is evidence that surface-volume factors may be active. More analyses are necessary before the explanation of the observed differences can be established.

IV. EFFECTS OF NUMBERS PRESENT ON DIVISION-RATES OF *Euglena gracilis* FREED FROM BACTERIA BY ULTRA-VIOLET IRRADIATION

Euglenae washed, as before, through baths A and B were irradiated[5] 2 and 3 minutes in these experiments and were then transferred by micropipette to sterile salt-solution cultures (as explained fully in Sec. II). Bacterial counts taken 10 minutes after transfer showed less than two colonies per 0.05 cc. by plate-counting methods (Table 1, B), and after 24 hours in culture even fewer bacteria were present. The method was, therefore, not any less effective in obtaining practically sterile cultures than the dilution method and involved one less transfer.

The results presented on the effects of irradiation on division-rates are those occurring because of the reactions of 2- and 3-minute irradiated organisms in sterile experimental environments when volumes and numbers of seeded euglenae are the only known variables. Every individual used in these experiments was irradiated in the same volume and with approximately one hundred other euglenae present during the exposure to ultra-violet.

The effects of irradiation for 2 and 3 minutes on populations of various sizes in 1 and 4 drops are shown in Figure 2. The numbers present in these series after 24 hours represent an ability to survive as well as to reproduce, and clearly reveal in these capacities differences which are dependent on the size of the initial population.

It is apparent at the outset that such comparisons as are shown in Figure 2 give evidence that growth of euglenae is not as great following irradiation as after treatment in dilution baths. Hence, we are dealing with an effect which is more harmful than that of washing.

The direction of increase in numbers produced per size of inocula is not the same in the irradiated as in the washed-sterile series, except with 2-minute irradiated euglenae in 4 drops where conditions are least toxic of any of the irradiated series. In 4 drops, washed-sterile euglenae divided significantly more rapidly than did 2-minute irradiated euglenae; in the cultures started with two individuals the value of P for the difference in rate was 0.0000; with four individuals in the inocula, 0.0000; with eight, 0.001; and with one and two considered together, 0.000.

The most harmful effects occurring in these series of irradiated euglenae are in those grown in 1 drop after 3 minutes of irradiation. Three-minute irradiation is decidedly more deleterious than that for 2 minutes, when the euglenae are cultured in either of the two volumes studied. In fact, in cultures in 1 drop and with irradiation for 3 minutes (Fig. 2, *c*) there is a population decrease in 24 hours in the cultures seeded with one, two, and four euglenae; and survival only is measurable. An increase in numbers present occurs only in the cultures with the largest number (eight) in the inocula. In this series an increase in density of initial population is, in every instance, of advantage: statistical

[5] A Cooper-Hewitt quartz mercury-vapor lamp was used; it ran at 110 volts D.C. and about 4 amperes, and at 66 cm. distance from the center of the arc to the culture being irradiated. It was over 10 years old and had been much used.

significance is attached to each difference in the numbers surviving or produced in cultures with successively greater numbers in the inocula. Table 3 gives the results of a comparison of the differences.

A significant advantage is also accorded larger-sized inocula in the group of experiments of 1 drop with euglenae irradiated for 2 minutes. The value for P for differences

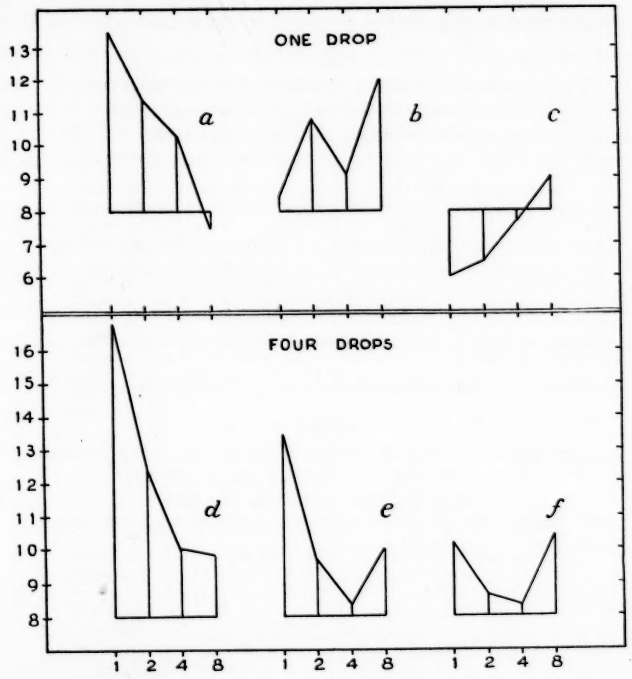

FIG. 2.—Differences in numbers of *Euglena gracilis* present after 24 hours in cultures inoculated with washed-sterile or 2- and 3-minute irradiated euglenae in 1 and 4 drops with varying numbers in the inocula. Ordinates, average total numbers present after 24 hours; abscissas, numbers in the inocula. *One drop:* (*a*) washed-sterile euglenae; (*b*) 2-minute irradiated; (*c*) 3-minute irradiated. *Four drops:* (*d*) washed-sterile; (*e*) 2-minute irradiated; (*f*) 3-minute irradiated.

between cultures started with two, as compared with one, individual is 0.04; for 4/1 is 0.0000; and for (4 and 8)/(1 and 2) P is 0.013.

In the larger volume, 4 drops, all cultures are capable of increasing their numbers; and the larger inocula are not significantly more successful than they were in the smaller volumes. Rather, the least numbers in the inocula have a relatively accelerated reproductive rate when only 2-minute irradiation is used, and the differences in the division-rates between smaller- and larger-sized inocula are significant (Table 4).

With euglenae irradiated for 3 minutes and cultured in 4 drops no difference is significant for any size of inocula, and the smaller inocula are not significantly retarded in

this volume. The larger volume is less deterring, therefore, to division of euglenae irradiated 2 or 3 minutes; and the final conditions approach those found in similar cultures started with washed-sterile individuals.

The fact that more harmful effects are seen in the 1-drop series, with either 2- or 3-minute irradiation, than in 4 drops, is in accord with the findings with the washed-sterile series in which optimal culture conditions seemed to prevail in 4 and 5 drops. However, in comparison of population densities in these two volumes, there are effects

TABLE 3

COMPARISON OF AVERAGE NUMBERS PRESENT WITH VARIOUS SIZED INOCULA OF EUGLENAE IRRADIATED FOR 3 MINUTES AND CULTURED IN 1 DROP

Comparison of Inocula Size	Average Total Numbers Present	Statistical Probability
2/1	6.5/6.0	0.000
4/1	7.6/6.0	0.02
8/1	9.0/6.0	0.0000
4/2	7.6/6.5	0.0051
8/2	9.0/6.5	0.0000
8/4	9.0/7.6	0.000
(1 and 2)/(4 and 8)	12.5/16.6	0.0000

TABLE 4

COMPARISON OF AVERAGE NUMBERS PRODUCED WITH VARIOUS SIZED INOCULA OF EUGLENAE IRRADIATED FOR 2 MINUTES AND CULTURED IN 4 DROPS

Comparison of Inocula Size	Average Total Numbers Produced	Statistical Probability
1/2	13.5/9.7	0.0098
1/4	13.5/8.3	0.0004
1/8	13.5/10.0	0.0092
2/4	9.7/8.3	0.0000
8/2	9.7/10.0	0.0098
8/4	9.7/8.3	.....
(1 and 2)/(4 and 8)	23.2/18.3	0.0032

on division-rate or on survival which indicate that some factors may be operating which are also dependent on the volume-per-individual phenomena.

In brief, under the conditions of these particular experiments, toxicity or deleterious factors in general, as manifested by lessened division-rates, are most significant in small volumes and when least numbers compose the inocula. As volume increases and/or numbers in the inocula increase, such factors show diminished effects.

V. THE EFFECTS OF NUMBERS INOCULATED ON RATE OF DIVISION IN CULTURES WITH SEVERAL SPECIES OF BACTERIA OF KNOWN CONCENTRATION

The following series of experiments were arranged in parallel fashion in order to ascertain the effects of symbionts and so compare the reproductive rates of *E. gracilis* in

the presence of mixed bacteria in known concentration with those given in bacteria-free inorganic media.

The media for these experiments were prepared in the following manner. Stock cultures were made in 100 cc. of the usual salt solution in small Erlenmeyer flasks. These were seeded with one or more pipettefuls of euglenae from the standard wheat-infusion stock and were well contaminated with bacteria of the wheat cultures. One cubic centimeter of supernatant fluid obtained by centrifuging 10 cc. of these salt-solution stock cultures was diluted with ten parts of sterile salt solution. Five species of bacteria were present in approximately the same proportion as found in the stock wheat infusions. The concentration of bacteria, as checked by colony-counting methods, was about 3,000,000 per cubic centimeter (Table 1, C). This approached the equivalent of a 0.001 X concentration suspension as used by Johnson (1933, 1936) and described in the next

TABLE 5

COMPARISON OF AVERAGE TOTAL NUMBERS PRODUCED IN NONSTERILE CULTURES INOCULATED WITH VARYING NUMBERS OF *Euglena gracilis* IN 1 DROP
(Average of twelve series)

Inoculated Number	Average Total Number	Comparison	Probability
1	6.3	1/2	0.14
2	8.75	1/4	0.0000
4	11.7	1/8	0.0000
8	10.75	2/4	0.08
1 and 2	7.75	2/8	0.048
4 and 8	11.2	4/8	0.18
		(1 and 2)/(4 and 8)	0.0000

section. These cultures will be designated as "nonsterile cultures," to avoid confusion with other bacterial suspensions referred to in Section VI.

In 1-drop cultures of the nonsterile series (see Table 5) euglenae divided more rapidly when seeded with two, four, or eight organisms than if but one was introduced. The results were similar to those obtained with 1-drop cultures of euglenae which had been irradiated for 2 minutes, and were opposite to those found using washed-sterile euglenae in the same volumes.

The cultures started with single individuals are most markedly altered in their division-rates in all these series: they show striking acceleration in the washed-sterile inocula and lack of maintenance of numbers in the nonsterile cultures and only a slight increase in the cultures inoculated with irradiated euglenae. Are the factors which accelerate division in small-volume cultures with smallest inocula of washed-sterile individuals absent in these nonsterile populations, or are they overborne by other factors?

Whatever the causal mechanism or mechanisms may be, the effect appears to be related to the inability of the smaller inocula, more especially of isolated euglenae, to flourish under the conditions that obtain in this so-called "nonsterile" series.

VI. COMPARISONS OF NUMBERS OF *Euglena gracilis* PRESENT AFTER 24 HOURS IN SUSPENSIONS OF CONTROLLED CONCENTRATIONS AND SPECIES OF BACTERIA

Each of the five species of bacteria which composed the natural flora of stock cultures of *E. gracilis* (either wheat or salt-solution stocks) was isolated on nutrient agar plates,

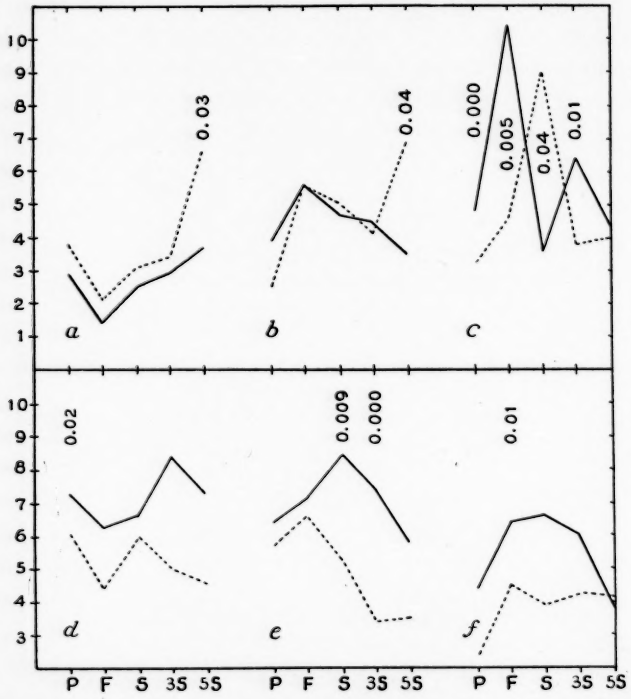

FIG. 3.—Differences in numbers of *Euglena gracilis* present after 24 hours in six concentrations of various bacterial suspensions in cultures (1 drop) inoculated with one and two (dotted lines) compared with four and eight (solid lines) washed-sterile euglenae. Ordinates, average total numbers present; abscissas, types of suspension. *P*, *Pseudomonas; F*, *Flavobacterium; S*, *Staphylococcus; 3S*, three-species suspensions; *5S*, five-species suspensions. When significant, the statistical probabilities of the differences are given (see legend to Fig. 1). The various figures show different concentrations of bacteria as follows: *a*, X; *b*, 0.1 X; *c*, 0.01 X; *d*, 0.001 X; *e*, 0.0001 X; *f*, 0.00001 X.

and a platinum loopful of a 24-hour colony of each species was transferred to 10 cc. of nutrient broth and incubated 24 hours. Then a loopful was transferred from the nutrient broth suspension to 10 cc. of sterile Osterhout salt solution to compose an X concentration of a known species of bacteria. Appropriate dilution gave a 0.1 X, a 0.01 X suspension, etc.

The bacteria used were *Flavobacterium*, a pigment-producing cocci, *Staphylococcus*,

sp., and *Pseudomonas fluorescens*. The species of the last genus was definitely established. Two gram-negative, non-spore-forming rods were also in stock cultures and were used in the cultures of the five mixed species. The three species suspensions were made up of *Flavobacterium*, *Staphylococcus*, and *Pseudomonas*. For the mixed cultures, of three and five species, loopfuls from three or five of the nutrient broth suspensions were placed in 30 and 50 cc., respectively, of sterile salt solution to compose X concentrations; and appropriate dilutions were made as indicated. Euglenae, in preparation for growth in

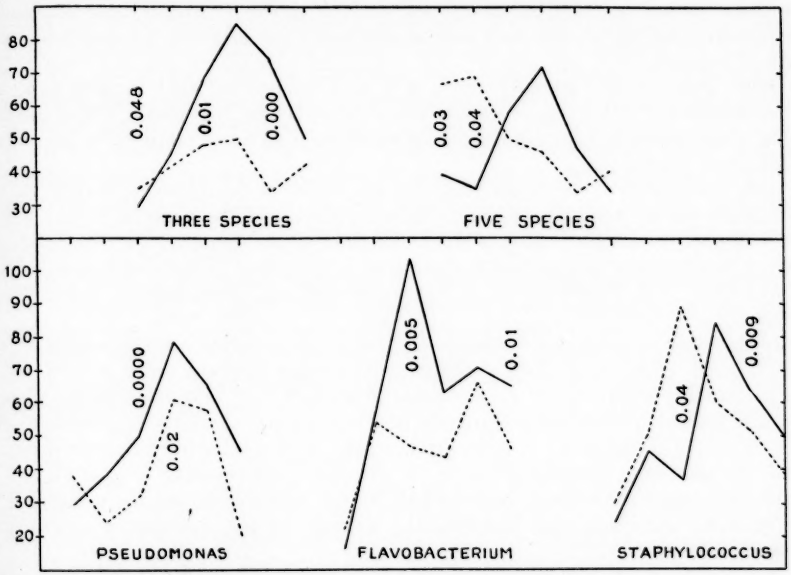

FIG. 4.—Differences in numbers of *Euglena gracilis* present after 24 hours in five bacterial suspensions (1 drop) of varying concentrations with one and two individuals in the inocula (dotted lines) compared with four and eight (solid lines). Ordinates, average numbers present; abscissas, concentrations of the suspensions for each pair of graphs; the points show the following concentrations of bacteria, X, 01 X, 0.01 X, 0.001 X, 0.0001 X, and 0.00001 X. When significant, the statistical probabilities of the differences are given.

these media, were passed through the three dilution baths and were mouth-pipetted to the final culture slide, as in all the washed-sterile series. All cultures were made in 1 drop of the standard salt solution.

Figures 3 and 4 schematize the cumbersome details accumulated in this series in a relatively simple fashion. The data from which these graphs are taken, together with the statistical calculations, are available, as are all the data for tables for previous sections (Sweet, 1937). Results from the cultures inoculated with one and two individuals have been considered together (dotted line), and, likewise, those inoculated with four and eight individuals (solid line).

Both figures present the same material but with different emphases. Figure 3 shows

the six concentrations separately, with the numbers of *Euglena* present after 24 hours in the five different types of bacterial suspension. Figure 4 presents the five different types of suspensions separately, with the numbers present in the various concentrations of each type of suspension. The two figures can be cross-read. The following summary of the material here presented uses both graphs.

1. There is a lack of maintenance of the numbers of *E. gracilis* inoculating a series in the majority of cases in these bacterial suspensions of different species and concentrations. In the sixty cases, only four instances occur in which an average of more than eight (the original inoculated for each series) individuals are present in these suspensions after 24 hours. Hence, for the most part we are measuring survival, not maintenance or growth of cultures.

2. From glancing at Figures 3 and 4 it is readily observed that in most cases the cultures inoculated with the greater numbers (four and eight individuals, heavy line) are capable of sustaining greater numbers than are those with fewer numbers in the inocula (one and two, dotted line).

3. This dominance of the larger-sized inocula occurs in nearly every instance in the less concentrated suspensions. (Refer to Figure 3, *d*, *e*, and *f*, the 0.001 X, 0.0001 X, and 0.00001 X concentrations.) This is true regardless of the specific type of suspension. However, this increase in numbers is significant in only four of the fourteen individual instances (Fig. 3, *d*, *Pseudomonas; e*, *Staphylococcus* and three species; and *f*, *Flavobacterium*.

4. In the more concentrated suspensions there is less uniformity in the results. In the 0.01 X concentration, three cases of significantly greater numbers occur in favor of the larger inoculating group, in the *Pseudomonas*, *Flavobacterium*, and three-species suspensions. The smaller inoculating group grow best in the *Staphylococcus* suspension at this concentration. Also in the 0.1 X concentration, the most significant difference occurs in favor of the smaller inoculating group in the five-species suspensions (Fig. 3, *b*).

5. In the most concentrated suspensions, X, the smaller inoculating group is consistently more numerous, but significantly so only in the five-species suspensions (Fig. 3, *a*).

6. In the most dilute and most concentrated suspensions—the X, 0.1 X, and 0.00001 X—numbers present in 24 hours are reduced more than in the moderately concentrated suspensions (Fig. 3, *a*, *b*, and *f;* cf. *c*, *d*, and *e*).

7. Hence, it is apparent that, where there are significant differences in the more concentrated suspensions, the smaller the numbers in the inocula the greater the chance of surviving; and in the less concentrated suspension, the larger the number in the inocula the greater the chance of survival. The transition occurs at the 0.01 X concentration.

8. In viewing these same relations in Figure 4, in order to note the specific effects of varying bacterial species in suspension and in a range of concentrations, no one type of supension is much different in effect than any other. In checking the average total numbers in each suspension, *Pseudomonas* is least conducive to survival, with an average of 4.55 living after 24 hours from an initial seeding of 8.0; the five-species suspension next, 5.01; the three-species, 5.11; *Flavobacterium*, 5.33; and *Staphylococcus*, sp., the most conducive, 5.41. These differences are, obviously, not great.

9. Significant differences in survival in each of the suspensions are not common.

a) In the *Pseudomonas* suspensions, statistically significant differences in numbers

present occur in the 0.01 and the 0.0001 X concentrations in favor of larger numbers in the inocula, although in all the concentrations of this type of suspension, except in the most concentrated, the larger number in the inocula are in advance of those with smaller numbers.

b) In the *Flavobacterium* suspension, again larger numbers do best in the concentrated suspension and are significantly more successful in sustaining *Euglena* in the 0.01 and the 0.00001 X; in fact, in the 0.01 concentration, increase in numbers occurs beyond the maintenance level.

c) In the *Staphylococcus* suspensions, only one significant difference occurs, and this in favor of the cultures with one and two individuals in the inocula in the 0.01 X concentration. In this group smaller numbers in the inocula are more numerous in the three most concentrated suspensions. In the more dilute suspensions the greater numbers are in advance—significantly so in one case, the 0.0001 X concentration.

d) In the three-species suspensions, three significant differences occur in favor of larger-sized inocula, in the 0.1 X, 0.01 X, and 0.0001 X concentrations. Only in the most concentrated suspensions do the smaller-sized inocula succeed in sustaining *Euglena* better than do larger-sized inocula; but the numbers are very reduced, and the difference is not significant.

e) And lastly, in the five-species suspension the significantly greater numbers occur in the X and the 0.1 X concentrations with smaller-sized inocula; and in the less concentrated, 0.0001 X concentration, with the greater-sized inocula.

Hence, in the eleven cases of statistically significant differences in the numbers surviving in 24 hours in such bacterial suspensions, seven of them occur in cultures with greater numbers in the inocula, three instances in the 0.01 X, one in the 0.001 X, two in 0.0001 X, and one in the 0.00001 X concentrations. The three cases in which smaller numbers in the inocula favor the survival of euglenae were in the more concentrated suspensions, X, 0.1 X, and 0.01 X. It can be said that, in general, in each of these specifically different bacterial supensions significant differences in survival are apparently related to the numbers seeded and are somewhat more assured in cultures with greater numbers in the inocula in the moderately dilute concentrations.

The division-rates in these bacterial suspensions, when viewed as a whole, are not as great as in any other of the series studied—washed-sterile, irradiated, or even nonsterile. In fact, usually maintenance is not possible: only survival-rates can be compared. There appears to be some deterrent of division present in practically all the concentrations, species, and combined species tested. However, because rates are not lower in at least one concentration of the five types of suspension, generally the 0.01 X or 0.001 X, than in some of the media already studied, there may be no factor except concentration of the specific bacterial suspension which is acting to lower rates.

In the five-species suspension, where the same species are present as in the stock and nonsterile cultures, it would be expected, perhaps, that in equal quantities the same or similar rates of division might obtain as in suspensions with similar proportions of the stock cultures. But numbers of bacteria present are considerably less. It appears that in equal quantities there may be some one or more species present in greater or less quantity than is conducive to growth of the culture in this short period. The *Pseudomonas* suspensions, the five- and the three-species suspensions showed least growth. Because *Pseudomonas* is common to these three least growth-promoting suspensions, there may be some effect of this species acting to deter division. More complete

study of the effect of *Pseudomonas* in mixed-population suspensions would be needed to reveal its symbiotic role; that is to say, the possible maximum or optimal numbers of *Pseudomonas* in a *Euglena* culture are not known from this limited survey.

Deterrence of division-rates was so universal as to lead one to suspect that, in the salt solution which was used, the presence of these bacteria react on *E. gracilis* adversely rather than beneficially. It is clear from these experiments that in the absence of some organic material, which is present in the wheat infusion, *Euglena* grows better in salt solution in the absence of bacteria than in the presence of the bacteria studied. The end-products of bacterial activity may not be absorbed, and the disintegration substances of dying and/or dead bacteria may be sufficient to limit division of *Euglena*.

In any event, as the numbers of introduced bacteria become smaller and conditions therefore approach those in sterile salt solutions, the effects of differential inoculation became similar to those found with the cultures in sterile salt solutions.

Also, whatever the true relationships are between *E. gracilis* and its associated bacteria in salt and wheat cultures, it is clear that in salt solutions and in the less concentrated cultures of many different types of bacterial suspensions, greater numbers in the inocula were more protective and effected greater survival. In the more dense suspensions the smaller numbers in the inocula sometimes show significantly greater numbers surviving. This latter effect may be related to the spatial factor found elsewhere in aggregation phenomena but not indicated especially in this population study heretofore. The former effect may be due to the greater capacity of increased numbers to counteract the toxicity of such bacterial suspensions in salt solution and of some of the toxic effects of plain salt solutions in large volumes of cultures made relatively free from bacteria by dilution methods as reported in Section III and by irradiation as shown in Section IV.

VII. THE EFFECTS OF NUMBERS ON SURVIVAL OF *Euglena gracilis* SUBJECTED TO ULTRA-VIOLET IRRADIATION

The methods employed for these cultures was that of transferring the desired number of euglenae after 20 minutes in dilution Bath C into open depression slides in 4 drops of sterile wheat solution, or bacteria-rich wheat solution, or into 1 or 4 drops of sterile salt solution. They were then subjected, as was described previously, for varying lengths of time to the same type of ultra-violet irradiation. As usual, results are based on counts made at the end of 24 hours. The solution for the sterile wheat was prepared by using boiled wheat after it had stood 24 hours, as described in Section II. The bacteria-rich wheat suspensions were similar to the type used in Section V with 1 cc. on the supernatant fluid of centrifuged *Euglena* wheat infusions mixed with 10 cc. of sterile wheat solution.

The protection of numbers of *E. gracilis* is apparent in the differences with which individuals are able to withstand the deleterious effects of long exposure to ultra-violet rays (Fig. 5). The presence of organic compounds and of bacteria definitely contributes to the survival-time of *E. gracilis* in sterile and bacteria-rich wheat infusions, particularly when ten euglenae are present and when twenty and forty are present through 6 minutes' exposure; beyond that time this differential was not present.

Cultures with single individuals in 4 drops of any of the three solutions were all killed after no more than $4\frac{1}{2}$ minutes' exposure. Those with ten euglenae survived the

6½ minutes' exposure in sterile wheat and bacteria-rich wheat solutions, whereas those in sterile salt solution survived no longer than did isolated individuals. Cultures with twenty individuals were able to live in the sterile salt solution after a 7-minute exposure; and in the wheat, sterile, and bacteria-rich, after an 8-minute exposure. Forty euglenae in 4 drops withstood exposures of 7½ minutes in sterile and bacteria-rich wheat solutions; in sterile salt solution the majority died at the end of 6 minutes' exposure, although a few persisted after 8 minutes. This is the only medium which allowed such survival after long exposure.

When euglenae are exposed in sterile salt-solution cultures of only 1 drop, the dele-

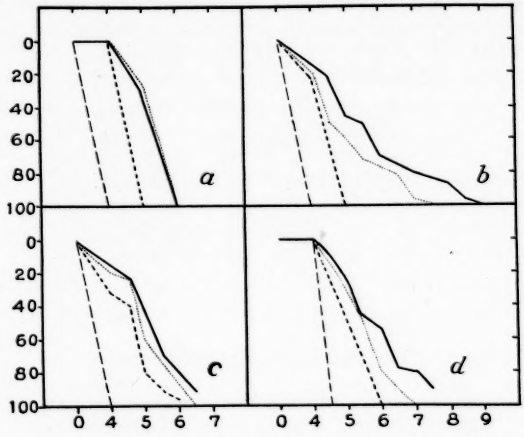

FIG. 5.—Average numbers of *Euglena gracilis* present after 24 hours, in three types of culture media, after exposure to ultra-violet irradiation of varying durations. (*a*) One drop, sterile salt solution. (*b*) Four drops, sterile salt solution. (*c*) Four drops, sterile wheat solution. (*d*) Four drops, bacteria-rich wheat suspension. Ordinates, percentage of loss; abscissas, duration of irradiation in minutes. . . . . = single euglenae; - - - - = ten euglenae; – – = twenty euglenae; —— = forty euglenae.

terious effect of irrradiation is greatest; and only cultures with twenty and forty organisms survive a 6-minute exposure; those inoculated with one or ten, only 4 minutes.

This small group of comparisons shows the advantage afforded greater numbers in any of these media in withstanding the effects of long exposure to ultra-violet irradiation and the relatively beneficial effects of organic media during all except the longer exposures. In the cases of twenty and forty euglenae in the bacteria-rich media, the presence of bacteria as an added protection against the toxicity of the rays is apparent. In the smaller volume of sterile salt solutions the deleterious effects are more drastic; even in these an increase in numbers of euglenae present insures more protection.

These relations cannot be explained on the basis of the amount of penetration, in so far as volume and bacterial effects are concerned. The presence of one or of forty euglenae in 1 or 4 drops of media, with or without bacteria or their end-products, would make very small difference in the penetration of the ultra-violet rays; hence, this effect is probably caused by some other set of relationships. Since the euglenae were cultured in the same solutions in which they were exposed to ultra-violet irradiation and since

irradiated water normally has an increase in ozone, it may well be that we are dealing here simply with mass protection from ozone. Euglenae are not particularly favorable organisms for a further analysis of the mechanism of this protection. Eliminating ozone as a factor, using the planarian, *Euplanaria dorotocephala*, Allee and Wilder (1939) have demonstrated mass protection from ultra-violet irradiation and have made some progress with an analysis of protective factors.

VIII. GENERAL DISCUSSION AND SUMMARY

Cultures in inorganic salt solution, almost or quite bacteria-free, in various suspensions of bacteria, in sterile and bacteria-rich wheat infusions, and of volumes ranging from 1 to 40 drops, have been used in this study of populations of *E. gracilis*. With numbers present at the end of 24 hours as an index, a variety of differences in division-rates of cultures inoculated with one, two, four, and eight individuals have been analyzed in order to find the effect of numbers used in initial seedings on survival and population growth. Constancy of light, temperature, humidity, pH, and in most cultures a strictly autotrophic media have been maintained. The controlled variables include, in addition to the number of euglenae inoculated, kinds and numbers of bacteria, volume, including surface area, and two methods of securing bacteria-free cultures. All the individuals used have been members of two clones between which and within which no measured differences in physiological states or division rates have been significant.

With such relatively small subcultures the results obtained indicate:

1. Greater rate of growth with smaller inocula:
   a) Washed-sterile euglenae in 1 (0.05 cc.) to 4 (0.20 cc.) drops
   b) Irradiated-sterile euglenae in 4 drops
2. Greater maintenance with smaller inocula:
   a) In bacterial suspensions
      (1) 0.01 X concentration, *Staphylococcus* suspensions
3. Greater survival with smaller inocula:
   a) In more concentrated bacterial suspensions
      (1) X concentration, five-species suspensions
      (2) 0.1 X concentration five-species suspensions
      (3) 0.1 X concentration

1. Greater rate of growth with larger inocula:
   a) Washed-sterile euglenae in 5 (0.25 cc.) drops
   b) Irradiated-sterile euglenae in 1 drop
   c) Nonsterile cultures of 1 drop
2. Greater maintenance with larger inocula:
   a) In bacterial suspensions
      (1) 0.01 X concentration, *Flavobacterium* suspensions
3. Greater survival with larger inocula:
   a) Washed-sterile euglenae in 10, 20, and 40 drops
   b) In more concentrated bacterial suspensions
      (1) 0.01 X concentration three-species suspensions
      (2) 0.01 X concentration, *Flavobacterium* suspensions
      (3) 0.01 X concentration, *Pseudomonas* suspensions
   c) In less concentrated bacterial suspensions
      (1) 0.001 X concentration, *Pseudomonas* suspension
      (2) 0.0001 X concentration *Staphylococcus* suspensions

(3) 0.00001 X concentration *Flavobacterium* suspensions
(4) 0.0001 X concentration, three-species suspensions

d) With exposure to toxic durations of ultra-violet irradiation
(1) In 1 and 4 drops of sterile salt solution
(2) In sterile wheat solution
(3) In bacteria-rich wheat suspensions

From these results in bacteria-free cultures it is apparent that density of inocula markedly affects growth and/or survival of populations of *E. gracilis*. Also, it seems clear that there exist optimal, suboptimal, and supraoptimal volume relations in cultures with washed-sterile euglenae which regulate growth or survival, and, also, that there are optimal, suboptimal, and supraoptimal densities of the bacteria associated with *Euglena*. For the most part, the mere presence of bacteria in a salt solution deters the development of euglenae. However, some materials or material are present in wheat media, even when autoclaved, which is accelerative to division-rates and which also affords protection against the toxic effects of ultra-violet irradiation.

Frequently during the experiments reported here, the numbers of individuals in the inoculum of a culture have made a significant difference in the rates of division and abilities to withstand environmental exigencies. As the foregoing summary shows, the smaller inocula have the advantage in a number of instances: with washed-sterile euglenae in 1 to 4 drops regardless of identical volume relations; in the cultures with larger volumes started with irradiated euglenae; and in the more concentrated bacterial suspensions. Stimulation due to the absence of members of the same species, and/or absence of a larger quantity of overly toxic substance or substances or of deleterious physical conditions, may determine the reactions of smaller numbers in the first instances. With the denser bacterial suspensions it is possible that survival is greater because of less competition in these more-dense mixed populations for space, light, acids, gases, or for certain of these.

Instances in which a larger inoculum has significant advantages over smaller inocula appear to be more numerous under the conditions tested. When the defects of an inorganic media accumulate, as in the volumes of from 5 to 40 drops; when toxic effects of irradiation are increased in smaller volumes, owing to greater evaporation, etc., as in the 1-drop versus 4-drop cultures; when time of exposure to ultra-violet irradiation is increased to a toxic amount; and when moderate and more dilute concentrations of various species of bacteria are present, greater numbers in the inocula insure cultures of having greater reproductive rates or greater chances of survival, or both.

There is no evidence from these experiments to indicate whether the irradiated-sterile, the washed-sterile, or nonsterile euglenae are most nearly normal. We know that in nature *Euglena* is an autotroph of stagnant, fresh-water, unshaded pools. In view of the type of reaction which occurs in the presence of mixed bacteria (of these relatively dense concentrations) and in the presence of a toxic agent (as represented by irradiation), protection as a result of the presence of greater numbers in the inoculum undoubtedly could occur in nature. Likewise, in the absence of bacteria, and with too great toxicity of the media, the accelerated division-rate of reduced numbers in the in-

oculum of *E. gracilis* may occur. That the types of population physiology shown in these cultures may exist in nature in a similar manner and not be limited to a few laboratory setups is not, therefore, unreasonable.

The experiments were not planned, however, to duplicate nature. They were conducted in the manner here recorded in an effort to bring the complex conditions to be found in an ordinary laboratory infusion under at least partial experimental control. These analytical experiments were attempted only after a long series of tests with more complex, and therefore less closely controlled, conditions which yielded indefinite, erratic results.

There have been described in this paper a number of situations, but there has been no attempt to analyze the causal factors at the base of such relationships as exist in these relatively simple experimental populations. The results of this study are relatively consistent within themselves and with the findings of others. Some of the complexities involved in this paper are, a priori, confusing. Errors in reasoning and in the conclusion based on this work may possibly be due to the natural tendency to simplify the categories and so avoid apparent inconsistency, in place of allowing many observations to dangle unrelated.

Similar work with protozoans has been done mainly with ciliates; and the relations obtaining in members of that group, fully discussed and reviewed by Allee (1931, 1934, 1935) and Johnson (1937), do not necessarily hold with an autotrophic organism. A discussion of the similarities and differences in results dependent on different physiological systems is reserved for the present.

The recent studies of Mast and Pace (1938) with the flagellate, *C. paramecium*, and of Reich (1938) with a soil amoeba, *M. palestinensis*, present additional substantiation of an allelocatalytic effect, whatever its basic factors may be. According to Reich (1938, p. 357), "cultures whose initial population was less than 0.03 amoebae per 0.1 cu. mm. of solution showed a distinctly lower rate of division in the first days than the controls with higher initial populations." And Mast and Pace (1938, p. 380) find "the rate of reproduction of *Chilomonas* increases to a maximum as the volume of culture fluid per individual decreases and then decreases to zero." The results obtained in various modifications of their experiments strongly indicate that *Chilomonas* secretes a substance which favors growth which they call *X*.

The only population study with a green flagellate that was available when the present paper was written was that of Jahn (1929), who found that the density of *E. gracilis* at the beginning of mass cultures which favor greatest growth was to be found in initial concentrations ranging from 38 to 39 organisms per cubic centimeter, rather than in those with a greater density of 350 to 894 organisms per cubic centimeter. In his work it is clear that "fewer" numbers in the inocula are most favorable in the early development of large quantity *Euglena* cultures.

It may be said that the conditions of his experiments were so different from those herein described as to make a comparison of results impossible.[6] And so it may be, but it is interesting to note certain similarities in results which do appear. If we analyze the terms "small" and "large" seedings in both series, we find a basis for comparison which may shed some light on Jahn's results and those of the present work.

[6] The composition of the media used by Jahn contained hydrolyzed casein and different inorganic salts; surface-area relations of mass cultures are different from those of depression-slide cultures.

In the present study of small-volume cultures the greatest concentration of organisms inoculating a culture was that of eight individuals in 1 drop, which is approximately 160 individuals per cubic centimeter. The concentrations which yielded greatest rates were those of one and two individuals per 4 drops (or five and ten organisms per cubic centimeter) and those of four and eight individuals per 5 drops (or sixteen and thirty-two organisms per cubic centimeter). The most dilute concentration of initial population was one organism per 40 drops or one-half organism per cubic centimeter. In other words, the greatest division-rates obtained in this range of small-volume cultures occurred in the moderately dense seedings. A comparison of Jahn's findings with a part of the data at hand (those with washed-sterile euglenae in salt solutions) is possible in this form.

A. Jahn's mass cultures — Cases in which less concentrated inocula gave a greater division-rate than more concentrated:

1. 89 individuals per cubic centimeter, compared with 890 individuals per cubic centimeter, showed a 10:1 division-rate
2. 55 individuals per cubic centimeter, compared with 555 individuals per cubic centimeter, showed a 3:1 division-rate

B. Present depression-slide cultures — Cases in which less concentrated inocula gave a greater division-rate than did more concentrated inocula:

1. 20 individuals per cubic centimeter (one individual per 1 drop), compared with 160 individuals per cubic centimeter (eight individuals per 1 drop), showed a 12.6:9.8 division-rate
2. 5 individuals per cubic centimeter (one individual per 4 drops), compared with 40 individuals per cubic centimeter (eight individuals per 4 drops), showed a 27.0:16.0 division-rate

Cases in which more concentrated inocula gave a greater division-rate than did less concentrated inocula:

1. 160 individuals per cubic centimeter (eight individuals per 1 drop), compared with 8 individuals per cubic centimeter (eight individuals per 20 drops), showed a 9.8:3.25 division-rate
2. 40 individuals per cubic centimeter (eight individuals per 4 drops), compared with 4 individuals per cubic centimeter (four individuals per 20 drops), showed a 10.3:1.25 division-rate

By combining the present findings with those of Jahn, it is possible to suggest that a supraoptimal density of initial populations of washed-sterile *E. gracilis* ranges above 80 individuals per cubic centimeter. An optimal size of inoculum apparently lies between 4 and 50 individuals per cubic centimeter, and a suboptimal one contains still fewer euglenae. This is a broad optimal range, to be sure; and it would not hold in each specific detail, particularly in depression-slide cultures, because there is a definite function of particular volumes, with an optimum at 4 and 5 drops in these experiments, regardless of initial population density. Jahn did not vary the surface area or volume of his mass cultures, so that relations which may depend on these are not brought to light.

Such a comparison as this indicates, at least, the necessity of using the terms "larger" and "smaller" with caution when referring to size of inocula, and of using, rather, the number of individuals per cubic centimeter wherever possible in drawing generaliza-

tions. In short, the densities of inocula which Jahn terms "light" are in the present work "heavy"; and the greatest division-rates occurred with approximately the same densities of initial population in both Jahn's mass cultures and the present series, in which small volumes are used.

Other functions or reactions of different numbers of the same species in the seeding of a culture appear in several ways in the data from small subcultures.

The data, as a whole, add a greater number of instances to the rapidly growing amount of verification of the principle of automatic co-operation among all plant and animal life than to the older and generally more easily proved interorganismic effects associated with overcrowding. Thus, these experiments lend additional support to the idea of the existence of an automatic mutualism, which Allee (1938) and his associates are investigating.

That signs of the beginnings of automatic co-operation and, to that extent, of a meager social organization appear in these unicellular and solitary chlorophyll-bearing organisms, whose status as members of the plant or animal kingdom is still in dispute, is not strange in view of the fact that colonial organization and even sexual differentiation are also to be found in the closely related Phytomonadida.

IX. CONCLUSIONS

1. Optimal, supraoptimal, and suboptimal volume relations of cultures of washed-sterile euglenae exist, which regulate growth or survival of a group accordingly.

2. There are optimal, suboptimal, and supraoptimal densities of bacteria associated with euglenae populations. Deterrence of division is common in the densities and with the species of bacteria tested.

3. Some material or materials present in sterile or nonsterile wheat infusions is more conducive to growth of populations of euglenae, and also affords more protection against toxic effects of ultra-violet irradiation, than does the artificial salt solution tested.

4. Smaller inocula show significantly greater advantage in situations in which the deleterious elements of the environment are not too great.

5. Larger inocula show significantly greater advantage when the defects of the environment are greatest.

6. Jahn (1929) found, from the study of mass cultures, no evidence of the Robertson effect; his least-dense cultures, however, correspond fairly well to the optimal numbers constituting an inoculum for the present small volume cultures, whose division-rates were found to be greater than either those in more dense or more dilute cultures.

7. The Robertson effect, or certain aspects of it, is present in some of the optimal volumes and may be operating in all of the supraoptimal volumes reported in this paper.

8. To this extent the present studies yield evidence of Robertson's effect. There is, however, no clear evidence of the validity of his theory.

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FLUCTUATIONS IN GROWTH-RATE OF EUGLENA ANABAENA, E. GRACILIS, AND E. VIRIDIS, AND THEIR APPARENT RELATION TO INITIAL DENSITY OF POPULATION

(Six figures)

R. P. HALL AND H. W. SCHOENBORN
Biological Laboratory, University College, New York University

IN STUDIES on autotrophic strains of *Euglena anabaena*, *E. gracilis*, and *E. viridis* in inorganic media the writers noted marked changes in the rate of population growth in successive transfers. In view of Dusi's (1937) observations that constant light was unfavorable for growth of certain Euglenidae, all our experimental lines were maintained at room temperature near an east window, and each transfer was incubated for 3 weeks. Although external conditions were fairly uniform for the different experimental series at any given time, both light and temperature varied from day to day. Since temperature and light were thus not constant during successive periods of incubation, variations in these conditions were at first suspected of inducing the fluctuations in growth-rate. This hypothesis was necessarily discarded when it was observed that the growth-rate was increasing in certain lines while decreasing in others. The pH of the medium was also eliminated as an important factor, since marked differences in growth-rates were observed in successive transfers showing the same initial and final pH values. The initial density of population also varied in successive transfers and it was noted that this factor showed certain relations to the growth-rate. A similar relationship has been described previously by Jahn (1929), who demonstrated that, in a pure line of *Euglena* sp., the rate of population growth was inversely proportional to the initial concentration of flagellates, particularly in cultures incubated for 10 days or longer. The observations described below are in general agreement with the findings of Jahn and show further that this relationship may be important in interpreting growth of a line through a series of successive transfers. The relationship between initial density of population and growth-rate has been traced in five different autotrophic lines, two of *E. gracilis*, one of *E. anabaena*, and two of *E. viridis*.

MATERIAL AND METHODS

Euglena anabaena var. *minor* (referred to here as "*E. anabaena*") and *E. gracilis*, in pure lines established by Mainx, were obtained bacteria-free from Professor E. G. Pringsheim in 1931; and a Mainx strain of *E. viridis* was obtained from the same source in 1937. Autotrophic strains of the three species have been established (Hall, 1938, 1939; Hall and Schoenborn, 1939) in inorganic media and have been utilized in the observations described below.

The following inorganic media were used:

Medium EAB		Medium EF	
NH_4NO_3	0.5 gm.	NH_4NO_3	1.0 gm.
KH_2PO_4	0.5 gm.	$MgSO_4 \cdot 7H_2O$	0.2 gm.
$MgSO_4 \cdot 7H_2O$	0.1 gm.	$CaCl_2 \cdot 2H_2O$	0.1 gm.
NaCl	0.1 gm.	KH_2PO_4	0.2 gm.
$FeCl_3 \cdot 6H_2O$	0.0025 gm.	$FeCl_3 \cdot 6H_2O$	0.0025 gm.
$MnCl_2 \cdot 4H_2O$	0.0001 gm.	$MnCl_2 \cdot 4H_2O$	0.0001 gm.
Distilled water	1.0 liter	Distilled water	1.0 liter

In allowing for the drop in pH during sterilization the reaction of each medium was adjusted with NaOH to pH 7.3–7.7. The medium was then tubed in measured amounts and sterilized in the autoclave at 15 pounds pressure for 20 minutes.

In the first transfer of every experimental series each tube received a 0.5-cc. inoculum from an autotrophic culture of the appropriate species. The tubes of the second transfer were inoculated in the same way from a tube of the first transfer after incubation, and so on in subsequent transfers. In each transfer approximately fifteen tubes were inoculated. Four of these were fixed for the initial count (number of flagellates per cubic centimeter), one was used for determination of initial pH, and the remainder were incubated for 3 weeks at room temperature near an east window. After incubation four tubes were fixed for the final count, one was used for determination of final pH, and another for inoculation of the next transfer. All pH determinations were made with a La Motte roulette colorimeter. Initial and final counts were made with a Whipple ocular micrometer and a Sedgwick-Rafter counting-cell. The amount of growth, expressed as x/x_0 (ratio of final to initial concentration of flagellates per cubic centimeter), was determined by a comparison of the final and initial counts.

Growth of *Euglena gracilis*

Series I in medium EF was followed for 33 weeks. Tubes of the first transfer were inoculated from an autotrophic culture of *E. gracilis* in the same medium at pH 6.6. The initial pH in the eleven successive transfers was: 6.9, 6.9, 6.9, 7.3, 6.9, 6.7, 6.9, 7.5, 6.9, 6.9, and 6.6. Each transfer was incubated for 21 days. Initial counts, growth, and final densities of population in the different transfers are compared in Figure 1. It will be noted that, for any two successive transfers except the sequence 7–8, a rise or fall in initial count is reflected inversely in the x/x_0 values. Transfer 8 showed both a low x/x_0 value and a small final population. These results may have been caused by a high initial pH in combination with a period of cold, cloudy weather in which the laboratory temperature was lower than that of previous periods.

Series II in medium EAB also covers a period of 33 weeks. The first transfer was inoculated from an autotrophic strain of *E. gracilis* in medium EAB at pH 6.7. Initial pH in successive transfers was as follows: 6.8, 6.9, 6.8, 6.7, 6.8, 6.7, 6.9, 7.0, 6.9, 6.9, and 7.1. The initial counts, x/x_0 values, and final densities of population are compared in Figure 2. The inverse relationship between growth and initial density of population is obvious in successive transfers.

Series III in medium EF was designed to compare cultures with different initial counts in regard to the relation between initial density of population and the amount of growth. In order to obtain a high initial concentration the contents of two tubes from transfer 8

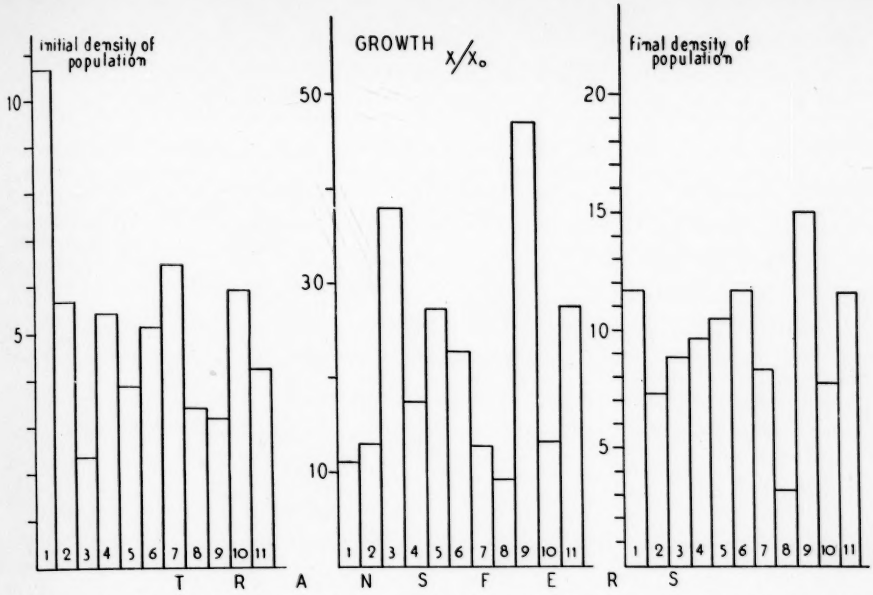

FIG. 1.—*Euglena gracilis*, series I. Initial density of population (hundreds per cubic centimeter), amount of growth (x/x_0), and final density of population (thousands per cubic centimeter) in eleven successive transfers.

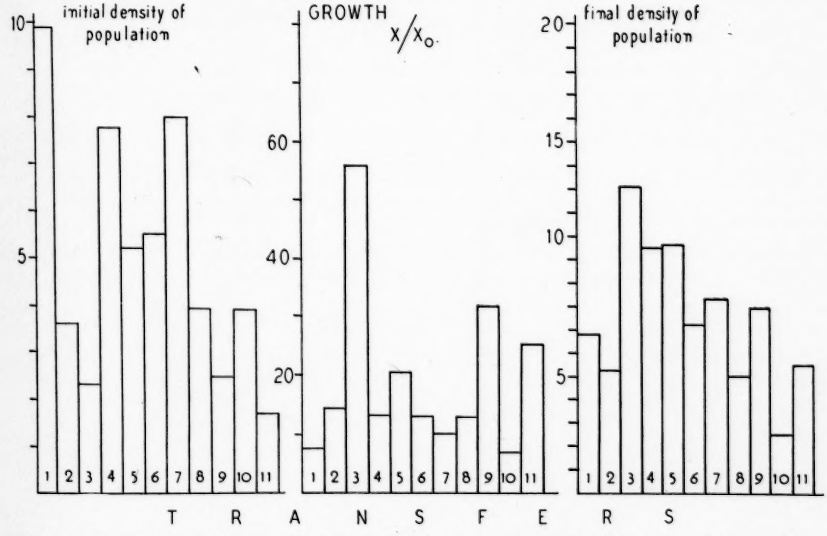

FIG. 2.—*Euglena gracilis*, series II. Initial density of population (hundreds per cubic centimeter), growth (x/x_0), and final density of population (thousands per cubic centimeter) in eleven successive transfers.

of series I were combined and used for inoculation (1.0 cc. of inocula) of the first transfer in series III. This transfer was inoculated at the same time as transfer 9 of series I and was identical with the latter with respect to type and pH of medium and time and other conditions of incubation. Any difference in rate of growth could, therefore, be attributed to the difference in inocula. In the second transfer, inoculations of 0.8 cc. were made from a tube of transfer 9 (ser. I); in all other respects transfer 2 (ser. III) was comparable to transfer 10 (ser. I). In the third transfer 2.0 cc. of material from a series I (transfer 11) tube were added to a tube of medium EF, which was used for inoculation (0.5 cc. of

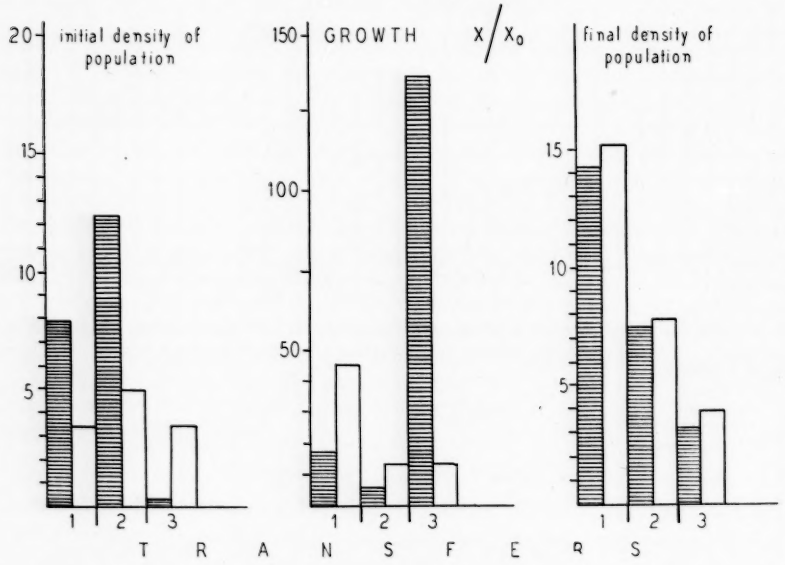

FIG. 3.—*Euglena gracilis*, series III. Transfers in series III (cross-hatched blocks) are compared with corresponding transfers in series I (white blocks) with respect to initial density of population (hundreds per cubic centimeter), growth (x/x_0), and final density of population (thousands per cubic centimeter).

inocula) of transfer 3 in series III. This procedure reduced the initial count for the third transfer to approximately one-fourteenth that for the corresponding transfer of series I. In Figure 3 the three transfers of series III are compared with the corresponding transfers of series I with respect to initial density of population, growth (x/x_0), and final density of population. In each transfer the inverse relationship between initial count and growth is apparent.

GROWTH OF *Euglena anabaena*

Series IV in medium EF covers a period of 24 weeks. The first transfer was inoculated from an autotrophic strain of *E. anabaena* in medium EF at pH 6.9. The initial pH in successive transfers was as follows: 6.9, 7.3, 6.9, 6.7, 6.9, 7.5, 6.9, and 6.9. In this series (Fig. 4) an inverse relationship between initial count and growth is apparent in every transfer.

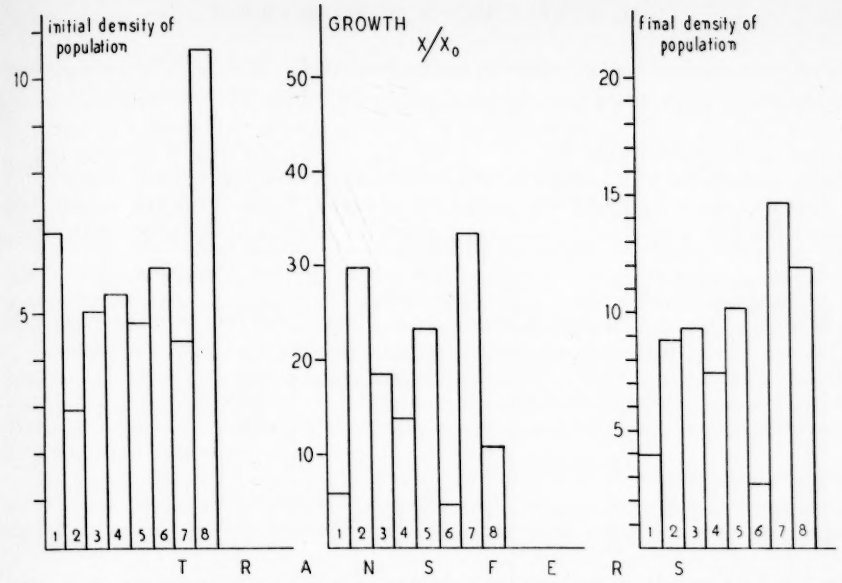

FIG. 4.—*Euglena anabaena*, series IV. Initial density of population (hundreds per cubic centimeter), growth (x/x_0), and final density of population (thousands per cubic centimeter) are compared in eight successive transfers.

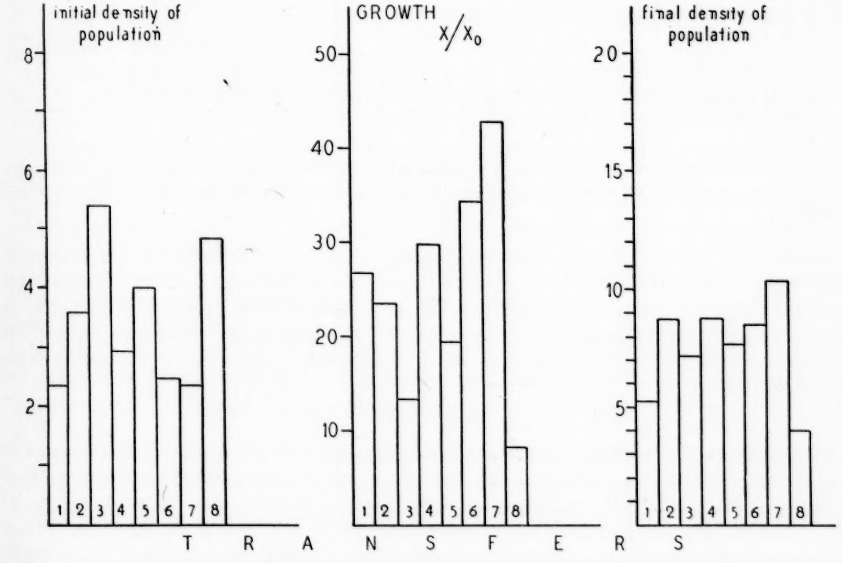

FIG. 5.—*Euglena viridis*, series V. Initial density of population (hundreds per cubic centimeter), growth (x/x_0), and final density of population (thousands per cubic centimeter) in eight successive transfers.

GROWTH OF *Euglena viridis*

Series V in medium EF was run concurrently with series IV. The first transfer was inoculated from an autotrophic strain of *E. viridis* in medium EF at pH 6.9. Initial pH in successive transfers was: 6.9, 7.3, 6.9, 6.7, 6.9, 7.3, 6.9, and 6.9. This series (Fig. 5) also showed inverse relationships between initial density of population and growth (x/x_0).

Series VI in medium EAB was carried for 24 weeks. Tubes of the first transfer were inoculated from a strain of *E. viridis* in medium EF at pH 6.9. Initial pH in successive

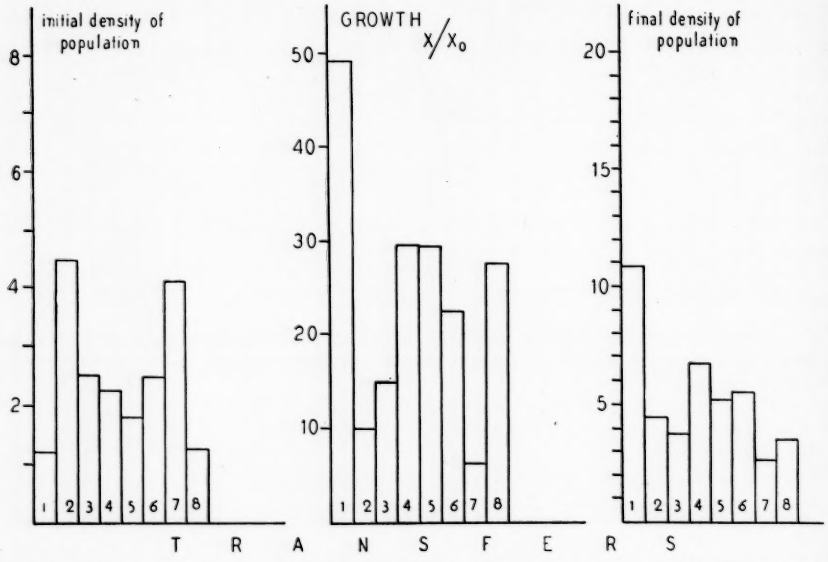

FIG. 6.—*Euglena viridis*, series VI. Initial density of population (hundreds per cubic centimeter), growth (x/x_0), and final density of population (thousands per cubic centimeter) in eight successive transfers.

transfers was: 6.7, 6.7, 6.7, 6.9, 7.0, 6.9, 6.9, and 6.9. In this series, as in the preceding ones, an inverse relationship between growth and initial count in successive transfers is apparent (Fig. 6) except in transfer 5. The x/x_0 values were practically identical in transfers 4 and 5, whereas greater growth was to have been expected in transfer 5. This fifth transfer overlapped in time transfer 8 in series I, where similar anomalies were observed.

DISCUSSION

On the basis of available information concerning growth of protozoan populations, it may be assumed that a population of *Euglena* in an inorganic medium increases to a certain maximal density (maximal stationary phase of growth) and then begins to decline. Preliminary observations have indicated that a population of *Euglena*, in our media, may approach the maximal stationary phase in cultures incubated for 3 weeks under favorable conditions. Under suitable environmental conditions a given culture

may approach the maximum quite closely, while a culture exposed to less favorable conditions will fall short of the maximum in a comparable growth period. Thus, differences in final population density were to be expected in different transfers of our experimental series, since the transfers were subjected to different environmental conditions. Such differences were observed (Figs. 1, 2, 4–6) and are probably to be attributed, in large measure, to variations in temperature and in intensity of light, factors obviously influencing the rate of population growth.

While environmental factors are undoubtedly important in determining the final density of population, it does not follow necessarily that the growth-rate is dependent upon these factors alone. Since the growth-rate (x/x_0), as the term is used here, represents the ratio of final to initial density of population, the initial density must also be considered as a factor. In our technique, as described above, the initial density of population for any given transfer is approximately one-twentieth the final density of population in the preceding transfer. Consequently, a large final population in one transfer would be followed by a high initial count in the next transfer, and a smaller population by a lower initial count.

The final density of population is obviously a function of the initial density and the growth-rate. If the rate of growth is approximately the same for a given set of environmental conditions, it must follow that a high initial count will give rise to a large final population and a lower initial count to a smaller population. However, there is no such correlation between initial and final densities of population in the results (Figs. 1–6) described above. As an alternative hypothesis, it may be supposed that, under the conditions of our experiments, a population of *Euglena* will approach the maximal stationary phase of growth, whether the initial count is comparatively high or comparatively low. If this is true, the growth-rate (x/x_0) will show an inverse relationship to the initial density of population.

The second hypothesis was tested in series III, in which paired transfers, differing only in inocula, were compared with respect to growth-rate and final density of population. In the first transfer (Fig. 3) the ratio of initial densities of population was 2.43:1; the corresponding growth-rates showed the inverse ratio of 1:2.5; and the final densities of population differed by 6 per cent, an amount not statistically significant. In the second transfer the corresponding ratios were 2.23:1 and 1:2.31, while the final populations differed by only 3.7 per cent. In the third transfer the ratios were 1:14.2 and 10.9:1. In this case, with a difference in initial counts much greater than in the other two transfers, the inverse relationship between initial density of population and growth-rate is less exact, although still apparent. The final populations (Fig. 3) in the various transfers differed appreciably; but in each transfer comparable populations were obtained in the paired series, irrespective of the initial count. These results indicate that for a given set of conditions, as specified above, the final density of population is, to a large extent, independent of the initial density, while the rate of growth shows an inverse relationship to the latter.

With due allowance for the fact that successive transfers in the various series were incubated at different times and hence were subjected to different conditions of light and temperature, the inverse relationship between growth and initial density of population should hold in all our experimental series. Such inverse relationships are apparent. For example, in successive transfers of series II these ratios are as follows: first and second transfers, 2.7:1 and 1:2.1; second and third, 1.5:1 and 1:3.8; third and fourth, 1:3.3 and

4.1:1; fourth and fifth, 1.5:1 and 1:1.5; fifth and sixth, 1:1.2 and 1.5:1; sixth and seventh, 1:1.5 and 1.3:1; seventh and eighth, 2.0:1 and 1:1.3; eighth and ninth, 1.6:1 and 1:2.4; ninth and tenth, 1:1.5 and 4.9:1; and tenth and eleventh, 2.2:1 and 1:4.0. With two exceptions, noted above (ser. I, transfer 8; and ser. VI, transfer 5), comparable relationships are evident in the other series. In general, when the final densities of population in successive transfers were approximately the same, the inverse relationship between growth-rate and initial density of population approached mathematical exactness. Several of the best examples are as follows: series I, fourth and fifth transfers, 1.4:1 and 1:1.5, and fifth and sixth transfers, 1:1.3 and 1.2:1; series II, fourth and fifth, 1.5:1 and 1:1.5; series IV, second and third, 1:1.7 and 1.6:1; series V, fifth and sixth, 1.6:1 and 1:1.7; and series VI, fifth and sixth, 1:1.37 and 1.28:1.

It is, of course, obvious that the growth-rate of *Euglena* in inorganic media is controlled in part by temperature and intensity of light, and it is important to determine whether variations in these factors could be primarily responsible for the fluctuations in growth-rate. This question may be tested by comparing the growth-rates in transfers of different series incubated during the same periods and under the same conditions. Thus, transfers 3–10 of series I correspond to transfers 1–8 of series IV and V. Similarly, transfers 4–11 of series II correspond to transfers 1–8 of series VI. A comparison of the corresponding growth-rates (Figs. 1, 2, 4–6) shows no correlation which can be attributed primarily to environmental factors. Hence, it appears that, even in transfers incubated at different times and under somewhat different environmental conditions, the initial density of population is more important in its influence on growth-rate than are various environmental factors which in themselves are known to affect the rate of population growth.

Such a relationship between growth-rate and initial density of population was clearly shown by Jahn (1929) in certain experiments with *Euglena* sp. in an inorganic medium, although his cultures were not bacteria-free. Our observations with bacteria-free cultures are in agreement with these findings of Jahn and show further that variations in initial density of population may bring about fluctuations in growth-rate of a strain during successive transfers in a given medium. Such fluctuations are, to a large extent, independent of environmental factors.

SUMMARY

Fluctuations in growth-rate of autotrophic strains of *E. anabaena* var. *minor*, *E. gracilis*, and *E. viridis* were noted in successive transfers in inorganic media. These fluctuations appear to be dependent upon an inverse relationship between growth-rate and the initial density of population. Under the conditions described, the initial density of population seems to be more important, in its influence on growth-rate, than are environmental factors which in themselves influence the rate of population growth.

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THE EFFECT OF TEMPERATURE ON THE SIZE OF THE COLUMNAR CELLS OF THE MID-INTESTINE OF THE JAPANESE BEETLE LARVA (POPILLIA JAPONICA NEWMAN)

DANIEL LUDWIG
Department of Biology, University College, New York University, and
Marine Biological Laboratory, Woods Hole, Massachusetts

ACCORDING to Titschack (1925), it is a general rule that insects reared at low temperatures grow to a larger size than those reared at higher temperatures. He discusses a few cases in which the reverse condition exists, and attributes the smaller size attained at lower temperatures to factors other than temperature. In many cases geographic races of a species exist in which the northern forms have been found to be smaller than southern forms of the same species. Titschack explains these cases as due either to differences in food eaten by the larvae or to a recent introduction of the species to a northern range in which temperature conditions are no longer optimal for development. Bělehrádek (1935), in his monograph on *Temperature and Living Matter*, discusses the effects of temperature on the size of cells and their constituents. He reviews about twenty papers recording observations on Protozoa, higher animals, and plants; and in every case larger cells were reported as found in organisms grown at lower temperatures.

Ludwig (1932) studied the growth of Japanese beetle larvae at temperatures of 20° and 25° C. He found that at all corresponding stages of development individuals were smaller when grown at the lower temperature. This smaller size of larvae, pupae, and adults at 20° might be associated with a smaller number of cells; or the cells themselves might be smaller in individuals grown at the lower temperature. In the present study, to determine which of these factors is responsible for the observed difference in size, the cells in the region of the mid-intestine limited by the second and third rings of gastric caeca were counted and measured in larvae grown at 20° and 25° C. These cells were selected for study because Abercrombie (1936) reported that, during the growth of the Japanese beetle, their increase in volume is proportional to the increase in size of the entire animal.

MATERIAL AND METHODS

Larvae of the Japanese beetle were obtained from eggs collected in the laboratory. As soon as hatched, they were isolated in 1-ounce metal salve boxes containing, as a medium, moist *Andropogon* mold. The salve boxes and the medium had been previously sterilized by heating for several hours at 150°–200° C. Several grains of wheat were placed in each box to serve as additional food for the larvae. They were then divided into two groups, one being placed in an incubator at 20° and the other at 25° C. Humidity was kept near the point of saturation by the addition of tap water to the medium; and food, in the form of both *Andropogon* mold and grains of wheat, was available for the larvae at all times

Larvae from each group were killed at stated stages of development at which studies on cell number and cell size were desired. The stages selected were the newly hatched

larva, freshly molted second-instar larva, and freshly molted third-instar larva. These stages were selected because each is probably of an equivalent age at the two temperatures and also because, immediately following the molt, less material is present within the alimentary canal to interfere with sectioning. When each larva had reached the desired stage, it was weighed, its body wall cut, and it was then immersed in hot Bouin's fixative. It was then dehydrated and cleared by the *n*-butyl alcohol method (Stiles, 1934) and sectioned in paraffin. Serial longitudinal sections of entire larvae were made at 5 μ thickness in the case of first- and second-instar larvae and at 7 μ thickness in the case of the third-instar larvae. They were stained with Delafield's hematoxylin and eosin. The progressive method of staining was employed.

The columnar cells of the mid-intestine were counted in median longitudinal sections of both the dorsal and ventral walls from the point where the epithelium begins to turn out into the second ring of gastric caeca to the corresponding point near the third ring of gastric caeca. The cells were measured with an ocular micrometer, using a 10 × ocular and a 1.8-mm. objective. In each larva typical cells were measured in the anterior, middle, and posterior regions of both the ventral and dorsal walls of the region studied. All counts and measurements were made with an oil immersion objective.

OBSERVATIONS

The results of the cell counts and measurements are given in Table 1. The average volume of each cell was obtained by multiplying the height by the width by the breadth of the cell, the latter having been earlier found to be equal to its width.

TABLE 1

EFFECT OF TEMPERATURE ON THE SIZE OF THE COLUMNAR CELLS OF THE MID-INTESTINE OF THE JAPANESE BEETLE LARVA

Stage	No. of Larvae Studied	Average Weight (Mg.)	No. of Cells in Ventral Wall	No. of Cells in Dorsal Wall	No. of Cells Measured	Average Width of Each (μ)	Average Height of Each (μ)	Average Volume of Each (Cu. μ)
	20° C.							
First instar (freshly hatched)	9	.....	146	208	53	3.66	33.97	455.05
Second instar (freshly molted)	12	13.1	217	344	72	6.20	30.48	1,171.65
Third instar (freshly molted)	9	49.0	432	660	54	6.08	50.40	1,863.10
	25° C.							
First instar (freshly hatched)	10	.....	147	208	59	3.87	29.43	440.77
Second instar (freshly molted)	11	17.5	226	353	66	7.03	32.05	1,583.94
Third instar (freshly molted)	9	79.7	443	693	54	7.26	45.61	2,403.99

Larvae reared at the lower temperature were smaller, the freshly molted second instars averaging 13.1 mg. at 20°, as compared with 17.5 mg. at 25° C. The corresponding weights for freshly molted third instars were 49.0 mg. and 79.7 mg. In spite of these differences in weight at the two temperatures, no significant differences were observed in the number of columnar cells in larvae of a given stage of development. An exact count of the cells in larvae reared at 20° was rather difficult because the cells were narrower and more crowded. The small differences shown in Table 1 are therefore not considered significant, since in no case do the numbers for a given stage differ by more than 5 per cent. Furthermore, the average numbers shown in this table for both 20° and 25° agree closely with those obtained at 25° C. by Abercrombie (1936) for larvae of corresponding stages of development.

Except in newly hatched larvae the columnar cells of the mid-intestine are definitely larger in individuals reared at the higher temperature. In freshly molted second-instar larvae the average cell volume was 1,171.65 cu. μ at 20° and 1,583.94 cu. μ at 25° C. These values are statistically different, since the difference is more than ten times its probable error. In freshly molted third-instar larvae the average volume of each cell was 1,863.10 cu. μ at 20° and 2,403.99 cu. μ at 25° C. In this case the difference is also more than ten times its probable error. In newly hatched larvae the columnar cells are approximately the same in size at both temperatures. This result was anticipated, since Ludwig (1932) found that the newly hatched larvae were the same in size, averaging 2.3 mg. at both temperatures.

The differences in size of the columnar cells of larvae reared at 20° and 25° C. are roughly proportional to the differences in weight of the entire animal. The newly molted second-instar larva at 25° weighed 1.3 times as much as at 20°, and the ratio of the cell volumes was also 1.3. For the newly molted third-instar larvae the ratio between the weights attained at the two temperatures was 1.6, and for cell size it was 1.3. Hence, the increase in size of larvae reared at the higher temperature is due, to a large extent, to the larger size of their constituent cells.

DISCUSSION

These observations and those of Ludwig (1932) indicate that the Japanese beetle is an exception to the general rule, enunciated by Titschack (1925), that insects attain a larger size when reared at a lower temperature. His explanations of exceptions to the rule do not seem to apply to this species. Titschack believed that, if a species grew to a smaller size at lower temperatures, some factors other than temperature are concerned. Among these, nutrition was considered very important. However, in these experiments with Japanese beetle larvae, food was carefully controlled, an abundance of wheat grains and *Andropogon* mold being available at all times at both temperatures. Titschack suggested that insects reared in the cold eat more food; and of the food eaten, more is absorbed and used for tissue synthesis. He performed some very exact experiments with the clothes moth, *Tineola biselliella*, which showed that at lower temperatures this species actually consumed more food and less fecal material was eliminated. While no exact comparison was made of the amount of food consumed by Japanese beetle larvae at 20° and 25° C., it was found that food had to be added much more frequently at the higher temperature. Because of the existence of a larval diapause, there is a relatively small difference between the duration of the larval stage at 20° and at 25° C. Ludwig (1928), under conditions similar to those in the present experiment, found its duration

at these temperatures to be 170 and 139 days, respectively. Hence, it is highly improbable that the amount of food consumed at the lower temperature would be greater.

Titschack believed that the recent spread of a species to a more northern climate might account for the smaller size of its northern races, since they are subjected to temperatures which are not favorable for development. If this idea is correct, we could divide insects into two groups, depending on their temperature reactions. One group includes northern species, which are adapted to cold climates and grow to a larger size when exposed to low temperatures; the other includes tropical or subtropical species, which are adapted to warm climates and which react unfavorably when exposed to low temperatures. However, in each case it is likely that heavier individuals are produced at temperatures which are optimal for the species involved. Because of its temperature reactions, the Japanese beetle belongs to the latter group. This idea is substantiated by the fact that, although the species is largely northern in distribution, the genus *Popillia*, to which it belongs, is predominantly tropical in distribution (Smith and Hadley, 1926).

The constancy in the number of columnar cells observed at both 20° and 25° C. indicates that temperature has little or no effect on cell number in the Japanese beetle. A number of studies have been made on the effect of temperature on the number of eye facets and the bristle number of *Drosophila melanogaster*. Seyster (1919) and Krafka (1919) reported that lower developmental temperatures resulted in a higher facet number in bar-eyed flies, and Luce (1926) found the reverse condition to exist in the case of infrabar. Eigenbrodt (1930) observed that the number of circumocular and ocellar hairs of this species varies inversely with temperature. Since each facet of an insect eye indicates the presence of a definite number of cells, and since the hairs are usually developed from single, modified, hypodermal cells, these studies indicate a change in the number of cells of the adult as the developmental temperature is changed. However, the results obtained on the Japanese beetle are not strictly comparable with those on *Drosophila*, since no observations were made on the cell number of adult beetles reared at different temperatures.

The observation that the columnar cells of the mid-intestine are smaller in Japanese beetle larvae grown at lower temperatures is opposed to the observations of many investigators (see Bělehrádek, 1935). Very little exact experimental evidence is available pertaining to the effects of temperature on the cell size of insects. Alpatov (1930) studied the size of the hypodermal cells of the wing of *Drosophila* grown at the temperatures of 18° and 28° C. The hypodermal cells were not measured, but their size was calculated by counting the number of hairs on a definite area of the wing. He found that the larger the wing, the smaller the number of cells on an area of 0.1 mm.^{2}, and consequently the larger the individual cells. Alpatov observed that the wings were larger in flies reared at the lower temperature. Hence, the hypodermal cells were also found to be larger at the lower temperature. It seems likely that the size of the insect is definitely correlated with the size of its constituent cells and that, irrespective of temperature, the larger individuals have larger cells.

SUMMARY

1. Japanese beetle larvae reared at 20° were found to be smaller than those reared at 25° C. The average weights of freshly molted second instars at each temperature were 13.1 mg. and 17.5 mg., respectively. The corresponding weights of freshly molted third instars were 49.0 mg. and 79.7 mg.

2. In spite of this difference in weight at the two temperatures, no differences were observed in the number of columnar cells in the mid-intestine of larvae of a given stage.

3. Except in newly hatched larvae, the columnar cells of the mid-intestine are definitely smaller in individuals reared at the lower temperature. Their difference in size is roughly proportional to the difference in weight of the larvae.

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THE RESPIRATORY METABOLISM OF THE NERVES OF THE BLUE CRAB (CALLINECTES SAPIDUS)

VERLUS F. LINDEMAN

Department of Zoölogy, Syracuse University, and Marine Biological Laboratory, Woods Hole, Massachusetts[1]

IN A recent study on the metabolism of the peripheral nerves of the lobster, Chang (1931) made the interesting observation that there was a difference in the oxygen consumption of the nerves of the claw as compared with the walking legs. Fragmentary data from numerous sources indicates that a similar difference exists in several other forms of Crustacea. The fact that there should be any great difference in the metabolic rate in two nerves of such similar morphological structure is in itself interesting.

In the present paper a study has been made of the oxygen consumption of the peripheral nerves of the blue crab (*Callinectes sapidus*). Data are presented which indicate a marked difference in the metabolic rate of the nerves of the walking leg and claw. Supplementary experiments are also reported which lend support to the belief that this is an intrinsic property of these nerves and not the result of physical factors in experimental procedure.

MATERIAL AND METHODS

The common blue crab, collected in the vicinity of Woods Hole, Massachusetts, was used throughout the study. The animals were collected daily by the staff of the Marine Biological Laboratory. No attempt was made to select the animals of the same size, but the average was about 6 inches across the carapace.

Two methods were employed in removing the nerves from the appendages. The first consisted of carefully dissecting the nerve from its position in the muscles. This proved at times very difficult because of the similarity of color between the nerves and the muscles, and often resulted in injury to the nerve. The second, and probably the most satisfactory, method was to break the appendage at the joint nearest the distal end and carefully strip the nerve from the inside of the remaining segments. As soon as the nerve was obtained, it was placed in several drops of serum, collected from the animal, and was immediately transferred to the respirometer.

The type of respirometer employed was a modified Fenn manometer. All experiments were carried out at a constant temperature in a bath provided for that purpose. After sufficient time had elapsed to insure equilibrium of pressures in the manometer, readings were made every 15 minutes until the experiment was complete. Most of the experiments required from 4 to 6 hours. The nerves were removed from the manometer, placed upon blotting-paper to remove excess moisture, and then immediately weighed. All computations were made in cubic millimeters of oxygen consumed per gram of moist nerve per hour.

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RESULTS AND DISCUSSION

The first two series of experiments consisted of measurements on the claw nerve at temperatures of 25°.8 C. and 20°.4 C., respectively. In both series paired nerves from the same animal were tested in separate manometers. In the first series eleven animals were used, and the results are presented in Table 1. Probably the most striking observation to be made from this table is the large variation in the oxygen consumption of the nerves from different animals. On the other hand, paired nerves of the same animal show a mean difference of about 4.5 per cent. Four different respirometers were used more or less indiscriminately throughout the course of the work, so that the large variation could hardly be attributed to faulty calibration.

TABLE 1

OXYGEN CONSUMPTION OF CLAW NERVE AT 25°.8 C.

Animal No.*	Manometer No.	Weight in Milligrams	O_2 Consumed per Gram per Hour
1 R	4	28	127.31
1 L	3	29	130.37
2, R	1	29	97.57
3 R	3	36	109.10
3 L	4	43	98.01
4, R	1	39	126.61
5 R	3	73	116.88
5 L	4	67	121.86
6 R	3	38	112.44
6 L	2	40	112.20
7 R	1	39	186.42
7 L	2	27	185.40
8 R	3	24	152.99
8 L	4	25	159.58
9, R	1	20	128.56
10 R	3	48	122.03
10 L	4	45	132.04
11 R	3	25	128.00
11 L	4	27	145.93
Average	.....	.....	136.40

* "R" indicates right appendage; "L" indicates left appendage.

This series of experiments was terminated when it became evident that a rapid increase in oxygen uptake occurred approximately 4 hours after the experiment was started. This, according to Shaffer, Chang, and Gerard (1935), is probably the result of bacterial action. In an attempt to delay this apparently abnormal increase in oxygen consumption a series was carried out at 20°.4 C. The results of this series are found in Table 2. Essentially the same results were obtained in this series as in series I except that the values for oxygen consumption were somewhat lower, which is to be expected in view of the lower temperature. The normal range of activity, however, was not greatly influenced by the lower temperature; and in no case was it possible to get constant readings for more then 5½ hours without a rapid increase in the oxygen uptake.

In the third series of experiments an attempt was made to compare the oxygen consumption of the nerves from the claw appendage and the first walking leg. Nerves from the same side were tested in separate manometers. The data presented in Table 3 show the results of eleven experiments, which are representative of a total of thirty. These data further emphasize the marked difference in the oxygen requirement in different

animals for both walking-appendage and claw nerves. Furthermore, a striking difference exists in all cases between the two kinds of nerves. The average oxygen consumption for the walking-leg nerve was found to be 156.10 cu. mm. per gram weight per hour, as compared to 99.09 for the claw nerve. These findings are not entirely in accord with

TABLE 2

OXYGEN CONSUMPTION OF CLAW NERVE AT 20°.4 C.

Animal No.	Manometer No.	Weight in Milligrams	O_2 Consumed per Gram per Hour	Animal No.	Manometer No.	Weight in Milligrams	O_2 Consumed per Gram per Hour
1 R	3	41	84.44	7 R	3	40	101.19
1 L	4	45	88.13	7 L	1	39	111.35
2 R	3	31	94.15	8 R	4	35	151.46
2 L	4	35	88.86	8 L	3	34	152.32
3, R	2	24	76.37	9 R	2	30	91.90
4 R	3	30	139.62	9 L	1	35	101.92
4 L	4	32	145.65	10 R	4	28	77.28
5 R	3	45	67.77	10 L	1	25	72.27
5 L	2	42	72.27	11, R	3	44	102.43
6 R	3	27	125.42	Average			104.74
6 L	4	25	150.10				

TABLE 3

OXYGEN CONSUMPTION OF THE CLAW AND WALKING-APPENDAGE NERVE AT 20°.4 C.

Animal No.	Cubic Millimeters of O_2 Consumed per Gram per Hour		Animal No.	Cubic Millimeters of O_2 Consumed per Gram per Hour	
	Walking	Claw		Walking	Claw
1	173.07	152.32	7	166.60	103.00
2	128.10	91.90	8	122.47	70.47
3	192.10	101.92	9	117.65	72.54
4	167.54	72.27	10	145.53	120.15
5	157.63	102.43	11	271.42	161.22
6	77.22	41.95	Average	156.10	99.09

those of Chang (1931), who showed that the walking-leg nerve of the lobster consumed less oxygen per hour than the claw nerve.

In an attempt to find a possible reason for this large difference in the metabolic rate of different peripheral nerves in crabs, a slight change in experimental procedure was devised. The possibility suggested itself that the claw nerve, being larger in diameter and thus having less surface in comparison to the mass, might allow slower exchange of gases

and therefore a slower oxygen uptake. In several experiments the claw nerve was split so that the diameter was approximately the same as the walking-leg nerves. The structure of the nerve lends itself well to separation, apparently with little injury. The data obtained were, in general, identical with the results obtained for the whole nerve. This, it would seem, tends to eliminate the factor of nerve diameter and indicates an intrinsic metabolic difference in the nerves of the claw and walking leg of the blue crab. This does not appear to be an unreasonable assumption in light of the results obtained by Guttman (1935) and Shapiro (1936), in which they demonstrated a regional difference in the metabolic rate within the same nerve, the optic nerve of the *Limulus*.

SUMMARY AND CONCLUSIONS

1. The oxygen consumption of the peripheral leg nerves of the blue crab (*C. sapidus*) was determined by means of a modified Fenn respirometer.

2. A wide variation in metabolism was obtained between the same nerves in different animals.

3. The oxygen consumption of the claw nerve was found to average 136.40 cu. mm. per gram weight per hour at 25°.8 C. and 104.74 cu. mm. per gram weight at 20°.4 C.

4. The metabolic rate of the walking-leg nerve was found to be about 56 per cent greater than the claw nerve.

5. The diameter of the nerve is apparently not an important factor.

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